A COMPARATIVE EVALUATION IN DIFFERENT TYPES OF TREATMENTS ON TITANIUM ALLOY SAMPLES WITH OR WITHOUT GENTAMICIN AND ITS EFFECT ON BIOFILM-AN INVITRO STUDY

Dissertation submitted to

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Certificate

This is to certify that the thesis entitled : **"A Comparative Evaluation In Different Types Of Treatments On Titanium Alloy Samples With Or Without Gentamicin And Its Effects On Biofilm- An In Vitro Study"** is a genuine work done by **Dr.Arun. R**, Postgraduate student during the period 2010-2013 under my guidance and supervision. The dissertation is submitted in partial fulfillment of the requirements for the award of Master of Dental Surgery, Branch I (Prosthodontics), The Tamilnadu Dr M.G.R Medical University, Chennai.

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LIST OF ABBREVIATIONS

HA	Hydroxy apatite
TiO2	Titanium dioxide
SEM	Scanning Electron Microscope
EDAX	Energy dispersive X-ray spectroscopy
EDS	Energy- dispersive X- ray analysis
CFU	Colony forming units
CpTi	Commercially pure titanium

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A comparative evaluation in different types of treatments on Titanium alloy samples with or without Gentamicin and its effect on Biofilm-An In Vitro study

ABSTRACT

Introduction

Use of Osseointegrated oral implants has been an excellent method for replacement of missing teeth. Biofilm formation on oral implants can cause inflammation of peri-implant tissues, which can affect the long term success of Osseointegrated implants. After exposure of implant to oral cavity, an acquired pellicle formed from salivary biopolymers becomes adsorbed to the implant surface. This pellicle forms the interface between the implant surface and initial microorganism like the *Streptococcus mitis, Streptococcus sanguis and Streptococcus oralis*. These bacterial microorganisms create a precondition for adhesion of periodontal pathogens such as *Haemophillus Actinomycetemcomitans, Porphyromonas Gingivalis, Prevotella intermedia, Treponema Denticola* which can induce peri implantitis with characteristic inflammation of the peri implant mucosa and destruction of the peri implant bone

Aims & Objectives:

To evaluate biofilm formation on various surface treated implants .

1. Comparative evaluation of Biofilm formation amongst 5 differently treated surface on Titanium samples.

2. To evaluate the difference in the delay of biofilm formation amongst various surface.

Methodology

Six set of polished Titanium samples was blasted with sintered HA and TiO2. Another set of samples were blasted and later loaded with Gentamicin drug by vaccum drying. All the samples are sterilized by autoclaving. The control group were plain polished and gentamicin drug loaded samples. All the samples were autoclaved and evaluation of the strains was done for biofilm. Bacterial adhesion was evaluated on time intervals of 0 hr,1 hr, 4 hrs 24 hrs and 48 hrs. Bio film was evaluated in colony forming units, and plotted against rough and polished samples.

Results

Analysis of variance ANOVA was the statistical tool employed to analyze the data.

1) All the samples showed bio film formation.

2) Bacterial adhesion was sequentially increasing in polished samples.

3) Initial bacterial adhesion was more on surface modified samples when compared to polished samples in the 1st hour

4) Bacterial adhesion was retarded in gentamicin coated HA blasted samples up to 24 hrs

5) Bacterial adhesion was considerably less on TiO2 blasted samples up to 48 hrs.

Summary

From the above findings it can be concluded that implant surface modified with TiO2 and gentamicin showed delayed biofilm formation even up to 48 hrs. These implants can retard the plaque formation thus prevents peri-implantitis in the primary healing stage. This in turn can prevent failure of implants. This is ideal in situations where the patient is having poor bone quality, poor oral hygiene and in patients suffering from debilitating disease.

Surface modification with HA has gained considerable osteoconductive surface which is a boon for the production of future implants with less expense, however further studies are to be carried out to prove its efficacy.

Pure titanium and titanium alloys are commonly employed as implant materials in dentistry due to their favorable combination of mechanical strength, chemical stability, and biocompatibility[1] .Commercially pure titanium (CpTi) has various degrees of purity (graded from 1 to 6). This purity is characterized by oxygen, carbon and iron content. Most dental implants are made from grade 4 CpTi as it is stronger than other grades[2]. Concept of Osseointegration by Brånemark of Sweden in 1952 paved the way for dental implants. The term refers to the direct structural and functional connection between living bone and the surface of a load-bearing artificial implant[3]. The integration of titanium implants with the surrounding bone is crucial for the successful bone regeneration and healing of a dental implant after surgical placement[4]. During the healing phase, platelets become activated after contacting with the implant surface and release a number of growth factors, such as platelet-derived growth factor (PDGF) and transforming growth factor beta (TGF-β) among many others and finally a new peri-implant vascular network starts to form.

In 1985, Dr. C. de Putter proposed two ways of implant anchorage / retention as mechanical and bioactive. Mechanical retention can be achieved by the topographical features in an implant like vents, slots, dimples, threads (screws) etc. which aids in the retention of the implant. Bioactive retention can be achieved in cases where the implant is coated with bioactive materials such as hydroxyapatite.

These bioactive materials stimulate bone formation leading to a physico-chemical bond.

Surface roughness of titanium implant

Surface roughness can be categorized as macro-, micro- and nano-sized topographies. The macro level is defined for topographical features as being in the range of millimeters to tens of microns. (more than 10µm). The micro topographic profile of dental implants is defined for surface roughness as being in the range of 1–10µm. Surface profiles in the nanometer range play an important role in the adsorption of proteins, adhesion of osteoblastic cells and thus it increases the rate of osseointegration[5]. A theoretical approach suggested that the ideal surface should be covered with hemispherical pits approximately 1.5µm in depth and 4µm in diameter. Various methods have been developed in order to create a rough surface and improve the osseointegration of titanium dental implants. These methods were titanium plasma-spraying, blasting with ceramic particles, acid-etching and anodization. The first generation of titanium implants had altered surfaces which improved the molecular interactions, cellular response and osseointegration.(6) The second generation of implant was made to accelerate and improve osseointegration by bioactive coatings and laser modified surfaces.(7)

On clinical evaluation the second generation implants were found to promote rapid bone formation by increasing osteo conductivity resulting in enhanced osteoblast and pre osteoblast adhesion.

Implant coatings improved stability during the healing process allowing immediate loading, especially in areas of poor bone quality and quantity [8,9]

Advantages of using Bio resorbable materials for grit blasting

Alumina (Al_2O_3) is frequently used as a blasting material and produces surface roughness but gets embedded into the implant surface and the residue remains even after ultrasonic cleaning, acid passivation and sterilization[2]. Alumina is insoluble in acid and is thus hard to remove from the titanium surface. Various In-Vitro studies have shown that Alumina particles get released into the surrounding tissues and may interfere with the osseointegration of the implants[10,11]. The second frequently used material for grit blasting is Titanium *oxide*(TiO2). Comparative clinical studies gave higher marginal bone levels and survival rates for TiO_2 grit-blasted implants than for machined turned implants[12]. A third possibility for roughening titanium dental implants consists in using a biocompatible, osteoconductive and resorbable blasting material such as hydroxyapatite, beta-tricalcium phosphate and mixtures. These materials are resorbable, leading to a clean, textured, pure titanium surface.[13,14] Experimental studies have demonstrated a higher bone-to-implant contact with these surfaces when compared to machined surfaces[13]

Soft tissue around implants(Peri-Implant mucosa)

The soft tissue surrounding healthy Osseo integrated dental implants shares anatomic and functional features with the gingiva around

teeth. An ideal peri-implant mucosa is lined by a stratified keratinized oral epithelium that is continuous with a junctional epithelium attached to the titanium surface by a basal lamina and by hemi desmosomes. A biological barrier of 3 to 4mm protects the zone of osseointegration during the healing phase of the implant. The peri-implant mucosa is similar to the gingiva around teeth as regards function and host response to infection[15,16]

Biofilm

After exposure to oral cavity, an acquired pellicle is formed which is from the salivary pellicle (consisting of salivary biopolymers) becomes adsorbed on all soft and hard oral tissues [17] . This is followed by growth-dependent accumulation by cell to cell adhesion to form a multilayer cell clusters in the polymer matrix[17] . The second step is irreversible adhesion caused by a time dependent shift to a higher binding affinity state, which involves multiple adhesions on the bacterial surface and polymer matrix increasing the Biofilm thickness. Biofilm layers are involved in a wide range of physical, metabolic and molecular interactions[18,19]. During the trans mucosal healing stage of implants, the adsorption of salivary pellicle and subsequent bacterial accumulation and biofilm formation induce an inflammatory process[20]. The composition of biofilm, speed of biofilm formation, surface energy, roughness, and chemical characteristics of the implant influence the formation of biofilm. The bacterial cells begin to colonize within 0-4 hours of

pellicle formation. A large proportion of initial colonizers are streptococci (S.Sanguis, S.oralis, S.mitis).the sequence of microbial colonization on dental implants and biofilm formation is similar to that of teeth[21,22] Streptococci were predominant after 4 hours, and anaerobes increase at 48 hours, which was common for all implant materials. This indicates that surface properties of implants influence early bacterial adhesion but not the bacterial flora or plaque maturation. Periodontal pathogens that bind to streptococci are the causative microbes for periodontal infections and peri-implantitis[23] .These pathogens produce endotoxins such as collagenase, hyaluronidase and chondroitin sulphates which trigger inflammatory response that results in loss of bone and periodontal tissues around the implant[24,25]. A recent in vivo study assessed the bacterial colonization on oral titanium implants immediately after placement and throughout the first 12 post-surgical weeks and compare the micro biota at interproximal, subgingival implant and adjacent tooth the study concluded that bacterial colonization occurred within 30 minutes after implant placement[25,26]. In fact, a biofilm is an accumulation of microbial cells within a matrix, optimizing the use of the available nutritional resources[27]. Various studies have demonstrated that both the quality and quantity of dental plaque adhesion on the implant surface are important in long term success of implants [28]

Antibiotic-loaded implant coatings

Antibiotic-loaded implant coatings are employed for the prevention of implant-associated infections. They can provide an immediate response to the threat of implant contamination but do not necessitate use of an additional carrier for the antibacterial agent. Antibiotics can be loaded on to the surface of implants by two ways one is by passive method and another by active method[29]. Passive coating technique aim to reduce bacterial adhesion by altering the physiochemical properties of the substrate so that bacteria-substrate interactions are not favorable. On the other hand Active coatings are designed for temporary release of high fluxes of antibacterial agents immediately following the implantation procedure. High local doses of antibiotics against specific pathogens associated with implant infections can thus be administered without reaching systemic toxicity levels with enhanced efficacy and less probability for bacterial resistance[30]. Gentamicin is an aminoglycoside antibiotic used to treat many types of bacterial infection. It is active against wide range of bacterial infection mostly gram '-ve' bacteria like the pseudomonas, proteus and gram '+ve' bacteria streptococcus and staphylococcus. **Gentamicin is one of the few heat stable antibiotics that remain active even after autoclaving,** which makes it particularly useful in the preparation of microbiological growth media[31].

Hence this study is a novel approach to evaluate the influence of bio film formation on surface modified implants with and without coating of gentamicin.

Aim

To evaluate biofilm formation on various surface treated implants .

Objectives

1. Comparative evaluation of Biofilm formation amongst 5 differently treated surface on Titanium samples.
2. To evaluate the difference in the delay of biofilm formation amongst various surface.

Introduction:

1. Rajesh et al on 2011 made a study on pulsed laser deposition of Hydroxy apetite on titanium interlayer. He came up with a conclusion that implants making use of HA coating can be further improved by providing an adequate inter layer of in vivo functionality and long term performance
2. Henry Martinez et al in the year 2001 made a review on optimal implant stabilization in low density bone. They concluded that primary implant stability is a fundamental factor in obtaining osseointegration. Clinical and radiographic evaluation of bone quality is essential. New implant surface textures and surgical protocol, designs have increased predictability in poor bone quality.
3. Jorg Nengebauer et al in the year 2009 did a study on mechanical stability of immediately loaded implants with various surfaces and design. A pilot study was done on dogs. The study investigates the biomechanical outcome of various designs and surfaces that claim to shorten implant treatment. The study came up with the conclusion that if a high primary stability can be achieved, then immediate loading is possible with various designs and surfaces.
4. Ahmed M. Ballo et al reviewed on dental implant surfaces such as its physicochemical properties, biologic performances and trends, it was summarized that the new generation dental implants exhibit a large variation in surface properties, both in terms of structural and chemical compositions. Future development of the next, third generation of dental implants should be based on increased knowledge about the interface

biology on cellular and molecular levels. He also added bacterial infection is a major challenge which may jeopardize the success of Osseointegrated implants; implant modification resulting in antibacterial activity should be given more importance.

5. Deepak V Kilpadi in the year 2000 made a study on cleaning and heat treatment effects on unalloyed titanium implant surfaces. The study concluded that ethanol cleaning of unalloyed titanium dental implants may not provide optimal surface properties when compared to cleaning with phosphoric acid followed by nitric acid passivation

Surface Roughness:

6. Anil etal reviewed on 2011 in dental implant surface enhancement and osseointegration. He concluded his review that topographical modification of implants has boosted the success rate of the implant therapy; especially in patients with poor bone quality sites and have significantly reduce the healing period.
7. L.LeGuehennec et al in the year 2005 made a study on surface treatments of titanium dental implants for rapid osseointegration. Osteoconductive calcium phosphate coatings promote bone healing and apposition, leading to the rapid biological fixation of implants. Surface treatments such as titanium plasma spraying, grit-blasting, acid etching, anodization or calcium phosphate coatings, and their corresponding surface morphologies and properties are described. The study concluded that surfaces with controlled and standardized topography or chemistry which would be the

only way to understand the interactions between proteins, cells and tissues and implant surfaces.

8. Alexandre-Amir Aalam et al in the year 2005 made a study on clinical evaluation of dental implants with surfaces roughened by anodic oxidation dual acid etched implants and machined implants. Seventy-four patients 198 dental implants were used. All 198 implants were radiographically and clinically successful. The implant size, location, bone quality, gender, age and smoking did not influence the comparative clinical outcome of the three groups. The study was concluded that with in the limitations of present study, Tillnite, Osseo lite and machined dental implants had similar short term clinical outcomes.
9. .Christine Hyon Foley et al in the year 2010 did a study on effect of phosphate treatment of acid etched implants on mineral apposition rates near implants in a dog model. He concluded that acid etched implants shows significantly higher mineral apposition rates compared to acid etched, phosphate –coated implants
10. Young-Taeg sul et al in the year 2007 made a study on optimum surface properties of oxidized implants for reinforcement of osseointegration. The study was intended to investigate detailed surface characterization of oxidized implants in a newly invented electrolyte system and to determine optimal surface oxide properties to enhance bone response. The study was concluded that surface properties of oxidized implants especially surface chemistry influenced bone responses.

11. Amarante ES et al in the year 2001 made a study on optimization of implant surfaces titanium plasma spray and acid –etched sand blasting. There is bone apposition onto the implant surface regardless of its characteristics polished or rough, made of titanium or ceramic. Roughness may play an important role in the percentage of bone apposition as well as in the velocity of apposition. The study concluded that besides optimizing the procedure, these surfaces characteristics may allow for an earlier loading of the implant and extend the indications for implants in low density alveolar bone and in regenerated bone.

12 Junker R et al in the year 2009 reviewed the effects of implant surface coatings and composition on bone integration. Their aim of was to evaluate the bone integration efficacy of recently developed and marketed oral implants as well as experimental surface alterations. It was concluded that surface roughening induces a safe and predictable implant-to-bone response, but it is not clear whether this effect is due to the surface roughness or to the related change in the surface composition.

13 Stanford CM in the year 2008 made a study on surface modifications of dental implants. The study was based on the impact of macro-retentive features which includes approaches to surface oxide modification, thread design, press-fit and sintered-bead technologies to increase predictabilities of outcomes. The study concluded that bone cells are exquisitely sensitive to these topographical features and will up regulates the expression of bone related genes for new bone formation when grown on these surfaces.

14 Reiner Mengal et al in the year 1998 made a study on the treatment of implant surfaces with different instruments. The aim of the study using implants and abutments was to examine traces left by various cleaning instruments and to determine the quantity of substance removal. It was concluded that cavitron jet air polishing system, the rubber cup, the plastic currete, Den sonic scaler with soft tip plastic fittings and titanium currete appear to b suitable for cleaning implant surfaces.

15 Marjorie K Jeff coat et al in the year2003 made a study on a comparison of hydroxyapatite-coated threaded, HA-coated cylindrical and Titanium Threaded Endosseous dental implants. The purpose of the study was to compare the success of hydroxyapatite (HA)-coated and machined titanium (Ti) implants in a 5 year randomized, controlled clinical trial each of 120 edentulous patients received HA-coated threaded, HA coated cylindrical, and machined Ti threaded implants in a randomized design using 5or6 months. The study concluded that over 5 years, the success rate tended to favor HA-coated implants.

16 Marco Morra in the year 2006 reviews the enhancement of bone regeneration at the interface with the implant device by immobilization of bio molecules to titanium surfaces. He concluded surface modification by ECM proteins appears as an effective way to stimulate bone regeneration over that provided by titanium

17 Ferguson S J et al in the year 2008 made a study on biomechanical comparison of different surface modification for dental implants. The purpose of the study was to evaluate biomechanical and micro

computerized tomographic osseointegration of implants to compare alternative, structural, chemical and biomechanical and pharmaceutical surface treatments applied to an identical established implant design. The study concluded that sandblasted and acid-etched titanium implant can be still be considered the standard surface for dental implants.

18 Klaus Gotfredson in the year 2001 did a study on fixed partial prosthesis supported by implants with machined and TiO2 blasted surfaces.He concluded that its showed good five year result with small ISFPP in the mandible, as well as in the maxilla.

Bio resorbable materials:

19 Buser D et al in the year 1999 made a study on interface shear strength of titanium implants with sandblasted and acid etched surfaces. The two best documented surfaces in implant dentistry, the machined and titanium plasma-sprayed (TPS) surfaces served as controls.. It can be concluded that the interface shear strength of titanium implants is significantly influenced by their surface charecteristics.since the machined titanium surfaces demonstrated significantly lower RTV compared with the TPS and SLA surface.

20 Osama A et al in 2000 studied on the effect of a hydroxyapatite – reinforced polyethylene stress distributor in a dental implant on compressive stress levels in surrounding bone. Stress breakers were

incorporated in to the dental implant which were HA reinforced polyethylene. The design lowered the compressive stress values in bone around the neck of implant

21 Hyeongil Kim in 2008 did a study on biocompatibility of SLA –treated titanium implants. The in vivo evaluation of SLA implant placed in rabbit tibia showed good bone to implant contact. In this short term study SLA implants demonstrated good clinical performance, maintaining good crestal bone height.

22 Rajesh et al in 2011 conducted a study on laser treated titanium substrate for pulsed laser deposition of highly adherent hydroxyapatite. In the study, ND-YAD laser beam of 355nm with 10Hz repetition rate was used for surface treatment of titanium as well as hydroxyapatite deposition. Based on the scratch test analysis and micro induction hardness values of coating, it was concluded that laser treated substrate has higher mechanical adhesion

23 Victoria Frojd in the year 2011 made a study on effect of Nano porous titanium oxide coating and anodized calcium ion modification of titanium surfaces on early microbial biofilm formation. Streptococcus sanguis and actinomyces naeslundi were used to investigate.. The study concluded that nano topographical modification of smooth titanium surfaces had no effect on adhesion or early biofilm formation by Streptococcus sanguis and actinomyces naeslundi as compared to turned surfaces or those treated with anodic oxidation in presence of calcium ion.

24 Jool Ong et al in the year 2002 made a study on Bone response to plasma sprayed hydroxyapatite and radiofrequency-sputtered calcium phosphate implants in vivo .The study concluded that the interfacial strength and histomorphometric data suggest that the cap coatings applied using the sputtering process produce bone responses similar to those of HA coatings applied using plasma spraying.

25 Gahlert M et al in the year 2007 made a study on biomechanical and histomorphometric comparison between zirconia implants with varying surface textures and a titanium implant. Surface analysis revealed the highest surface roughness for the SLA implants, followed by ZrOZr and ZroZr.the study concluded that ZrOZr can achieve a higher stability in bone than ZrOZm implants. Roughening the turned zirconia implants enhances bone apposition and has a beneficial effect on the interfacial shear strength.

26 Giovanna Orsini et al in the year 2000 made a study on surface Analysis of machined versus sandblasted and acid etched titanium implants. Cytotoxicity tests showed that sandblasted and acid etched implants had non cytotoxic cellular effects and appeared to be biocompatible. SEM examination showed that the surface roughness produced by sandblasting and acid etching could affect cell adhesion mechanisms. It was concluded that these morphologic irregularities could improve initial cell anchorage, providing better osseointegration for sandblasted and acid etched implants.

27 Adriano Piattelli et al in the year 1998 made a study on histologic and histomorphometric analysis of the bone response to machined and sand

blasted titanium implants. The aim of the study was to make a comparative analysis between the bone response to machined and sandblasted implants. Under SEM examination, machined implants presented typical machining grooves while rough, irregular surface with depressions was present on sandblasted implants. It was concluded that sand blasted showed higher osteoconductivity as a result of higher surface roughness.

28 Hak-Kwan Kim et al in the year 2004 studied on surface modification of implant materials and its effect on attachment and proliferation of bone cell. They concluded that surface topography may be a key factor in determining the morphological and functional responses during the osteoblast-substrate interactions

29 P A Ramires et al in the year 2001 made a study on influence of titania/hydroxyapatite composite coatings on invitro osteoblasts behavior .they concluded that these biomaterials are very promising and a better understanding of cells/bio material interaction and mechanisms can help in the development of more dental implants.

30 Su-Hee Lee et al in the year 2006 studied on hydroxyapatite-TiO2 hybrid coatings on Ti implants.the results suggest that the HA precoating of Ti prior to MAO treatment can be beneficial to its hard tissue implants

Soft tissue around implants:

31 Haruyuki Kawahara et al in the year 1998 made a study on biological seal of titanium implants concentrating on the epithelialization mechanism around the dental implants .This study concludes that difference in the

growth, contact, and adhesive strength of the HGE and HGF cells to Ti surfaces promotes apical epithelialization under the pathologic condition.

32 Byung-chul Lee et al in the year 2011 made a study on initial bacterial adhesion on resin, titanium and zirconia in vitro.He concluded his study that resin specimen showed the roughest surface and have a higher susceptibility to adhere streptococcus sanguis than titanium and zirconia. There was no significant difference in bacterial addition between titanium and zirconia in vitro

33 Grossner – Schreiber et al in 2011 did a study on plaque formation on surface modified dental implants, an invitro study. He came up with a conclusion that physical modification of titanium implants surfaces such as coating with TiN or ZrN may reduce bacterial adherence and hence improve results.

34 Marc Quirynen et al in 2002 reviewed infectious risks for oral implants, they quoted that longevity of Osseointegrated implants can be compromised by occlusal overload or plaque induced peri implantitis depending upon implant geometry and surface characteristics. Micro biota in perimplantitis is same to that of periodontitis. Finally periodontitis enhancing factors such as smoking and poor oral hygiene also increase the risk of peri implantitis

35 Clark M Stanford in the year 2010 reviewed surface modifications of biomedical and dental implants and the processes of inflammation wound healing and bone formation. He concluded the complex role of implant surface topography and impact on healing response plays a major role in

biologic criteria that can guide the design and development of future tissue- implant interface.

36 Sami Rossi et al in the year 2008 studied on peri implant tissue response to TiO2 surface modified implant.He concluded that nano porous sol-gel derived TiO2 thin film on ITI Straumann dental implant improved soft tissue attachment in vivo.

Biofilm:

37 Birte Grobner-Schreiber et al in 2001 did an invitro study on plaque formation on surface modified dental implants. they evaluated the influence of two physical hard coating on bacterial adhesion in comparison with control surface of equivalent hardness. They concluded that physical modifications of titanium implant surface such as coating with TiN or ZrN may reduce bacterial adhesion and hence improved clinical results

38 Antonoio Scarano et al studied on bacterial adhesion on commercially pure titanium and zirconium oxide disks, they concluded that zirconium oxide may be a suitable material for manufacturing implant abutments with a low colonization potential.

39 Groessner-Schreiber B et al in 2004 studied whether different implant surfaces exposed in the oral cavity of humans shows different biofilm compositions and activities. He studied on the influence of two physical

hard coatings on bacterial adhesion was compared with pure titanium surface. He concluded that implant coatings to decrease peri implant soft tissue inflammation.

40 Rajiv Saini in the year 2011 reviewed on oral biofilm and dental implants. Biofilms forms under fluid conditions. Dental plaque is an example for host – associated biofilm. Dental plaque is a classic example of both a biofilm and microbial community. Future control and treatment of biofilm research will affect the success rate of dental implants.

41 Angie lee et al in the year 2011 reviewed biofilm related to dental implants by discussing biofilm around dental implants, peri implant health, and transition from health to peri implant diseases. They concluded reduction of the bacterial load to level compatible with health is an important factor of implant therapy.

42 Sebastian Grade et al in the year 2011 studied on structural analysis of *in situ* biofilm formation on oral implants. He used 15 titanium healing abutments were inserted in 6 patients for 14 days. Biofilm was stained with fluorescent Live/ Dead Backlight kit before examination by confocal laser scanning microscopy. Results showed thickness was ranging from 0 and 80 µm. thi9s method uniquely describes an effective way to depict biofilm development on implant surface in both supra and gingival regions.

43 Anna G et al in the year 2011 did a study on streptococcus sanguis adhesion on titanium rough surfaces by the effect of shot-blasting particles. From this work they concluded that physicochemical properties

of particles used for surface modification to titanium surface lead to different bacterial adhesion. Alumina shot- blasted titanium surface, presented a lower amount of bacteria attached, compared to silicon carbide shot-blasted surfaces.

44 Cornelius Elter et al in the year 2008 did a study to establish a noninvasive method for quantitative analysis of supra and sub gingival biofilm formation on dental implants considering different surface modifications.. Biofilm evaluation was done using SEM, including secondary- electron and Rutherford back scattering detection methods. Biofilm accumulation was significantly higher in supra gingival compared to sub gingival. The study concluded saying that significant influence of surface localization as well as surface modification on biofilm modification.

45 Heinrich W.S Tillman et al in the year 1998 made a study on evaluation of three different dental implants in Ligature-Induced peri implantitis .The purpose of the study was to evaluate peri implant breakdown microbiologically, radiographically and histologically. Hydroxyapatite-coated, titanium plasma-sprayed, and titanium alloy surfaces were investigated. It was concluded that all implants were equally susceptible to peri –implantitis.

46 Karthekayen Subramanian et al in 2009 did a review of literature on biofilm in dental implants. He concluded saying increase in surface roughness and surface free energy facilitates biofilm formation on dental implants and abutment surface. Surface chemistry and the design features

of the implant abutment configurations also played a significant role in biofilm formation.

47 W.Heuer et al in the year 2007 made a study on analysis of early biofilm formation on oral implants in man. They investigated biofilm formation on oral implant abutments and to check whether Haemophillus Actinomycetemcomitans and P.Gingivalis were present in the crevicular fluids around oral implants. The study concluded that absence of periodontal pathogens from the sulcus fluid during initial bacterial colonization.

48 Herles et al in November 1994 conducted a study on chemo stat flow cell system. The study developed on experimental in vitro model of dental plaque to assess the potential efficiency of anti-plaque agents. The mixture was pumped through six flows cells, each containing two types of surfaces on which plaque formed and was subsequently measured. The quantity of plaque formed on both types of surfaces gradually increased with the duration of flow.

49 Almagner- Flores et al on 2011 did a study on influence of topography and hydrophilicity on initial oral biofilm formation on micro structured titanium surface in vitro. He came up with a conclusion that initial biofilm formation and composition were affected by the media used.

50 H.J. Busscher et al in the year 2010 reviewed biofilm formation on dental restorative and implant materials. They have critically discussed modification of implant surface with biomaterials to discourage biofilm formation. It has concluded that for dental applications, antimicrobial

coatings killing upon contact are more promising than antimicrobial releasing coating.

51 Wim Teughels et al in the year 2006 made a study on effect of material characteristics and/or surface topography on biofilm development. The objective of study was a systemic review aimed to evaluate critically the impact of surface characteristic on biofilm formation. The study was conducted that the trans mucosal implant surfaces with higher surface roughness/surface free energy facilitates biofilm formation.

Antibiotic loading:

52 Gabriel Colon et al in the year 2006 did a study on increased osteoblast and decreased staphylococcus epidermidis functions on Nano phase ZnO and TiO2.they suggest that Nano phase ZnO and TiO2 may reduce S.epidermidis adhesion and increased osteoblast functions.

53 Llinos G Harris et al in the year 2008 reviewed on implant coatings to prevent infection. He concluded to date no coatings fully prevents bacterial adhesion.

54 XU Ming-fang et al in the year 2006 did a study on characteristics of nano structure of N-TiO2 thin films and photo bactericidal action. The results indicated that the photo induced bactericidal efficiency of N-TiO2 thin films probably dependent on the characteristics of the film

Materials and Methodology

In the present study an effort was made to find out the influence of biofilm on surface modified implants with and without coating of gentamicin.

Materials:

1. Titanium samples (ASTMF11O8, MANHER METAL SUPPLY CORPORATION, Mumbai, India.)
2. Sintered hydroxyapatite
3. Sintered TiO2
4. Gentamycin (Magenda, Wockharditt, Bangalore)
5. Microbial strain *Streptococcus sanguis* (ATCC strains distributed by Hi media labs Mumbai)
6. Titanium dioxide (Analytical rasayan from Aldehydes chemical, BioSar, India)
7. Phosphate buffer saline 1 X
8. Ringer solution
9. Acre dine Orange (S0006)

Equipments:

1. Sandblaster (oxymeter, Manfredi, Italy)
2. Sintering furnace (OKAY, Raising-Health, electric furnace, SAKH and co, Calcutta)
3. Grinder and polisher (Beuhler- Eco met 3 variable speed grinder and polisher, Beuhler ltd USA)
4. Isostatic pressing machine (Xpress, SPEX sample prep, USA)
5. Pulverizer (Janke and Kankel, IKA- labor technique, Staufen Italy)

6. Tumbling machine (Retsch, Retsch GmbH and co.kg Germany)
7. Ultrasonic cleaner (EUMAX ultrasonic cleaner)
8. Scanning Electron Microscope(SEM) (ESEM- Quanta 200, Germany)
9. Energy dispersive X-ray spectroscopy EDAX(EDAX; OXFORD, X-Ray microanalysis software)
10. Vacuum dryer (Heraseus Vaccutherm, Germany).
11. Auto clave (Confident pvt Ltd Company, Bangalore, India)
12. Centrifugal machine (Eppendroff ,India ltd)

Armamentarium:

1. In house gauge and blasting gun holder
2. Sieves (Retsch, Retsch GmbH and co.kg Germany)
3. Micro polish(Beuhler- gamma micro polish, alumina no 3 Beuhler ltd USA)
4. Test tubes
5. Centrifugal tubes (Eppendroff tubes)
6. Petri dish
7. Tissue culture plates (Tarson, Kolkata, India)

Preparation of Ti samples:

Commercially available Ti6Al4V (ASTMF11O8,MANHER METAL SUPPLY CORPORATION, Mumbai India) was machined to 2mm thickness and 2 × 1.5 cm length and breadth rectangular samples. These discs were mechanically polished by silicon carbide papers of grit size 240 and 600 in the grinder and polisher

(Beuhler- Eco met 3 variable speed grinder and polisher, Beuhler ltd USA) to have a uniform surface. These samples were cleaned ultrasonically and further cleaned with distilled water, rectified spirit and Acetone followed by drying in hot air.

Preparation of sintered hydroxyapatite powder:

HA powder was prepared in house by a wet precipitation technique using CA (No3)2-4H2O (calcium nitrate) and NH4 H2 PO4 (ammonium di hydrogen phosphate) (Ranken India) at PH 11 and 80 °C. The calcined mass was sintered at 1100°C for 2 hrs. The sintered HA blocks were tumbled (Retsch, Retsch GmbH and co.kg Germany) with ceramic balls and were made to fine powder. The fine powder was sieved (Retsch, Retsch GmbH and co.kg Germany) to obtain uniform particle sizes in the range of 65µm, 125µm and 250 µm.

Preparation of sintered titanium di oxide:

Commercially available titanium di oxide (Analytical rasayan from Aldehydes chemical, BioSar, India) of molecular weight 79.89 was used. The micro fine powder was compacted at 200 Mpa in a cold isostatic press (Xpress, SPEX sample prep, USA) and subsequently these pressed blocks were sintered at 1100°C for 2 hrs. The sintered blocks were pulverized into fine powder. The pulverized powder was sieved to uniform particle sizes in the range of 65µm, 125 µm and 250 µm.

Surface modification by blasting:

A brand new blasting machine was used to avoid contamination of alumina powder which was used in the existing one. A gauge was deviced with acrylic sheets with gradations starting from 1cm- 10cm. The gauge could also hold the

gun in the desired position it was intended to be. Provision was given to hold the target sample disc firmly in position. The gauge could be easily kept inside the blasting machine. The pressure was maintained at 40 psi.

HA powder was loaded in the jar of the blasting machine of particle size (1) 65µ (2) 125µ (3) 250 µm. On each particle size the target samples were away from the gun distance 2cms, 4cms, 6cms. Each sample was blasted for 2 minutes 4 minutes and 6 minutes. 5 samples of each particle size, distance and time was blasted.

The same procedure was carried out for TiO2 also. The jar of the machine was emptied and cleaned to avoid contamination. The samples were blasted with TiO2 only after the complete elimination of HA particles from the blasting chamber to avoid contamination with HA. The titanium samples blasted with HA and TiO2 were now cleaned ultrasonically and dried. The center of the blasted area was evaluated for surface roughness under scanning electron microscope (SEM). Elemental study was done on the above surfaces with the help of EDAX.

Scanning electron microscopy and energy- dispersive X- ray analysis(SEM-EDS)

The coatings obtained were compared with the targets for the elemental composition and microstructural morphology by an environmental scanning electron microscope (ESEM- Quanta 200, Germany) equipped with an energy dispersive X-ray analysis device (EDAX; OXFORD, X-Ray microanalysis software). The samples were examined as such at low vacuum mode without any coating.

Anti-microbial drug loading:

After evaluating the surface topography another group of surface modified samples were prepared for gentamicin loading. Gentamicin (magenda, Wockharditt, Bangalore) injection vials were used. All the samples were autoclaved. The samples were transferred to a sterile test tube which contained gentamicin drug. The drug was loaded to the samples by vaccumizing with the help of vacuum forming dryer (Heraseus Vaccutherm, Germany). The samples were vacuumed for 15 minutes at 200mbar. Further the samples were transformed to sterile petri glass dish and vacuum dried for 15 minutes at 200mbar in room temperature inside the vacuum forming dryer. The samples were having white color patches over the surface which showed that drug was loaded and dried on the surface. All the samples were transferred to a sterile petry dish and were autoclaved at 121˚c for 15 minutes at 15 psi.

Bio film evaluation:

Isolates from fresh agar plates were inoculated with streptococcus sanguis and incubated for 18 hour at 37^{o}C in stationary condition and diluted 1in100 with fresh medium. Titanium samples (5 samples of each group) were transferred in to Eppendorf tubes containing 1ml streptococcus sanguis cultures. Bacterial concentration was about 10^{9} ufc/ml. They were incubated for 0hr, 1hr, 4hrs, 24hrs, and 48hrs at 37^{0} c. To quantify the bacteria adhered to the samples, the samples were washed twice with PBS1X and then it was introduced in to new Eppendroff tubes containing 1ml of ringer solution. The samples were vortexed on the centrifugal machine for 5 minutes. Viable counts in the supernatant were determined by staining with Accredine Orange S 0006(0.1 %w/v) under

microscope. Live CFU's were quantified and normalized as CFU's/ $mm^{2.}$ The viable count was plotted against roughness and plain samples.

Table-1: Number of viable organisms in plain polished titanium

Sample Number	0 hour	1 hour	4 hour	24 hour	48 hour
1	0	22	65	84	110
2	0	21	60	86	113
3	0	20	62	83	109
4	0	24	61	81	107
5	0	20	65	84	110
6	0	21	60	86	112
(MEAN±SD)	0.00±0.00	21.33±1.50	62.17±2.32	84.00±1.89	110.17±2.14

Table-2: Number of viable organisms in plain polished titanium+ Gentamicin

Sample Number	0 hour	1 hour	4 hour	24 hour	48 hour
1	0	16	52	73	98
2	0	14	50	70	98
3	0	16	49	71	93
4	0	13	54	75	96
5	0	15	51	70	94
6	0	16	53	72	98
(MEAN±SD)	0.00±0.00	15.00±1.26	51.50±1.87	71.83±1.94	96.17±2.23

Table-3: Number of viable organisms in HA blasted

Sample Number	0 hour	1 hour	4 hour	24 hour	48 hour
1	0	30	58	79	90
2	0	29	55	83	95
3	2	33	60	77	87
4	0	30	56	76	89
5	0	32	56	79	92
6	0	34	58	78	94
(MEAN±SD)	0.33±0.82	31.33±1.97	57.17±1.83	78.67±2.42	91.17±3.06

Table-4: Number of viable organisms in HA blasted+ Gentamicin

Sample Number	0 hour	1 hour	4 hour	24 hour	48 hour
1	0	28	40	61	73
2	0	32	42	62	70
3	0	26	42	63	68
4	0	30	40	63	75
5	0	30	38	58	73
6	0	28	42	61	70
(MEAN±SD)	0.00±0.00	29.00±2.01	40.67±1.63	61.33±1.86	71.50±2.59

Table-5: Number of viable organisms in TiO_2 blasted

Sample Number	0 hour	1 hour	4 hour	24 hour	48 hour
1	0	34	46	70	81
2	0	30	44	70	84
3	0	36	48	74	83
4	0	37	48	68	79
5	0	32	43	68	80
6	0	36	48	72	82
(MEAN±SD)	0.00±0.00	34.17±2.71	46.17±2.23	70.33±2.33	81.50±1.87

Table-6: Number of viable organisms in TiO_2 blasted+ Gentamicin

Sample Number	0 hour	1 hour	4 hour	24 hour	48 hour
1	0	20	28	39	52
2	0	20	28	43	50
3	0	19	30	38	50
4	0	18	30	36	56
5	0	21	28	36	53
6	0	22	29	39	50

(MEAN±SD)	**0.00±0.00**	**20.00±1.41**	**28.84±0.98**	**38.50±2.59**	**51.83±2.40**

Table 1 shows number of viable organs in plain polished samples.

Table 2 shows number of viable organisms in plain polished titanium with gentamicin coated.

Table 3 shows number of viable organisms in HA blasted samples.

Table 4 shows number of viable organisms in HA blasted with gentamicin coated.

Table 5 shows no of viable organisms in TiO2 blasted samples.

Table 6 shows viable organisms in TiO2 blasted samples with gentamicin coated.

Statistical Analysis

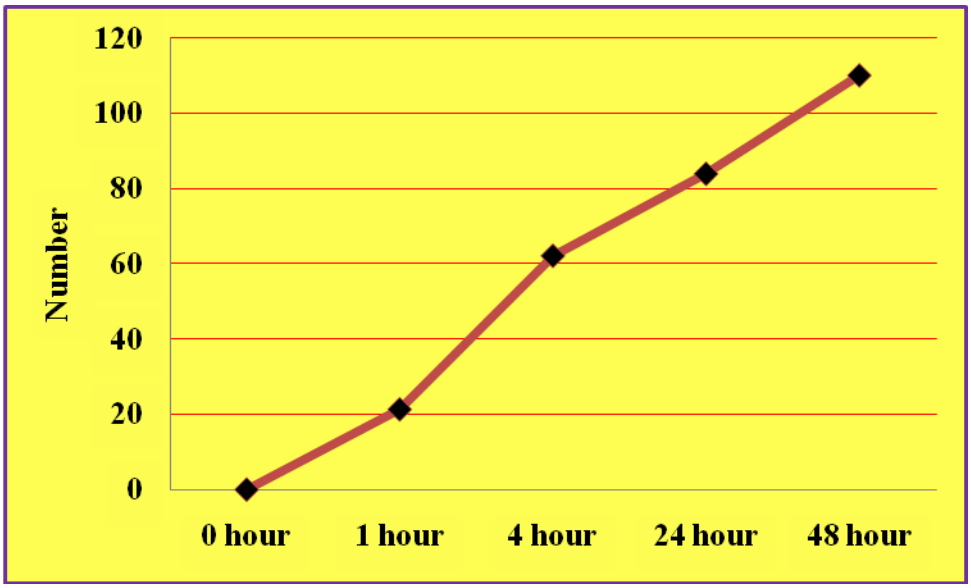
The data was analysed by SPSS software 16.0 version. ANOVA was applied for comparing between the groups. Post hoc test followed by Dunnet t test was used to find the significant difference at 95% confidence interval. $P<0.05$ between the groups considered statistically significant.

Groups	**Composition**
Group-I	Plain Polished Titanium (PPT)
Group-II	Plain Polished Titanium (PPT) + Gentamicin (PPTG)
Group-III	HA Blasted Surface (HA)
Group-IV	HA Blasted Surface+ Gentamicin (HAG)
Group-V	TiO_2 Blasted Surface (TBS)
Group-VI	TiO_2 Blasted Surface + Gentamicin

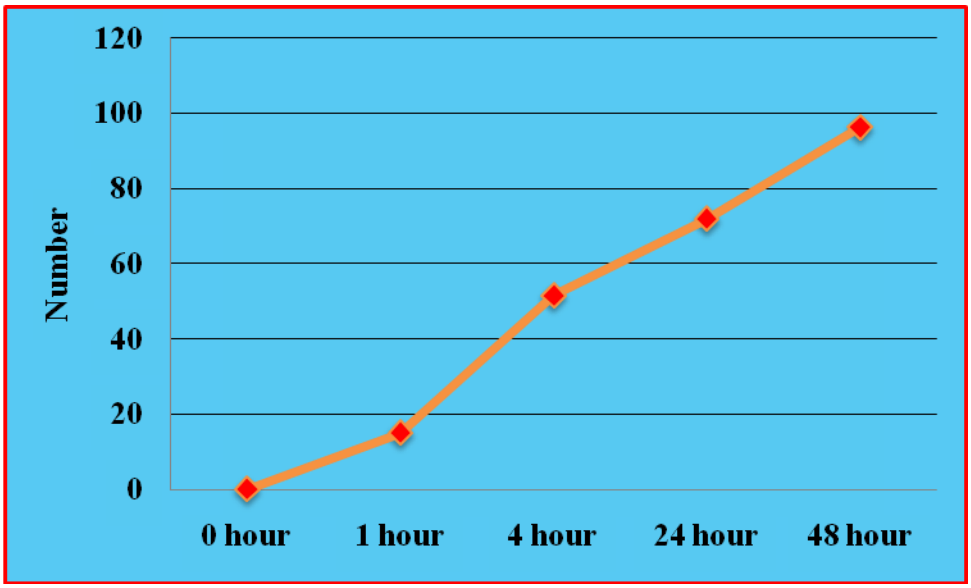
Table-1: Mean values of number of viable organisms of different groups

Groups	**O hour (MEAN±SD)**	**1 hour (MEAN±SD)**	**4 hour (MEAN±SD)**	**24 hour (MEAN±SD)**	**48 hour (MEAN±SD)**
Group-I	0.00±0.00	21.33±1.50	62.17±2.32	84.00±1.89	110.17±2.14
Group-II	0.00±0.00	15.00±1.26	51.50±1.87	71.83±1.94	96.17±2.23
Group-III	0.33±0.82	31.33±1.97	57.17±1.83	78.67±2.42	91.17±3.06
Group-IV	0.00±0.00	29.00±2.01	40.67±1.63	61.33±1.86	71.50±2.59
Group-V	0.00±0.00	34.17±2.71	46.17±2.23	70.33±2.33	81.50±1.87
Group-VI	0.00±0.00	20.00±1.41	28.84±0.98	38.50±2.59	51.83±2.40

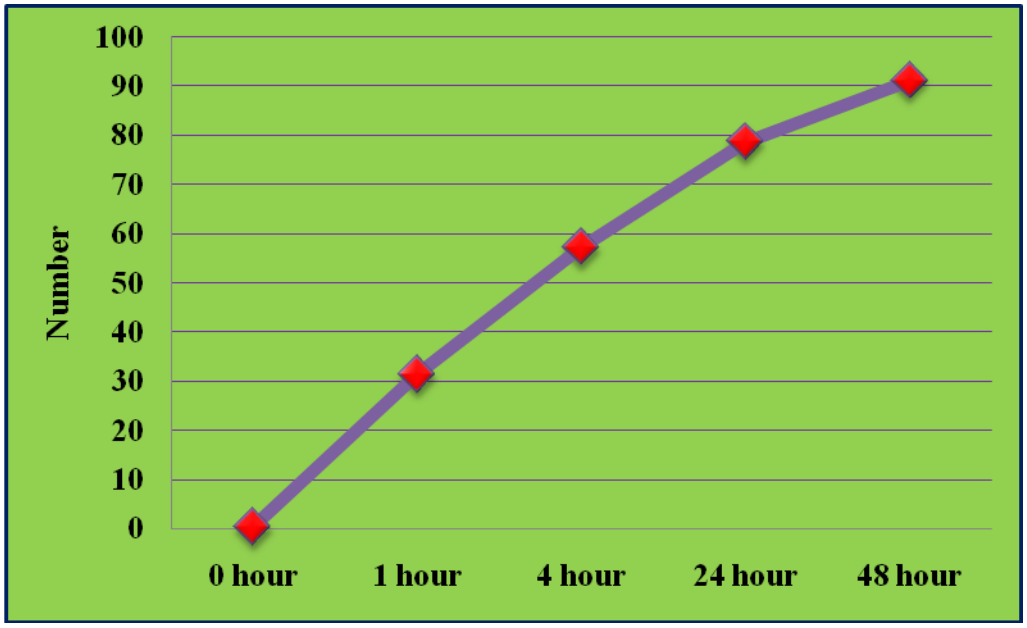
Grpah-1: Mean values of number of viable organisms in group-I at different time interval



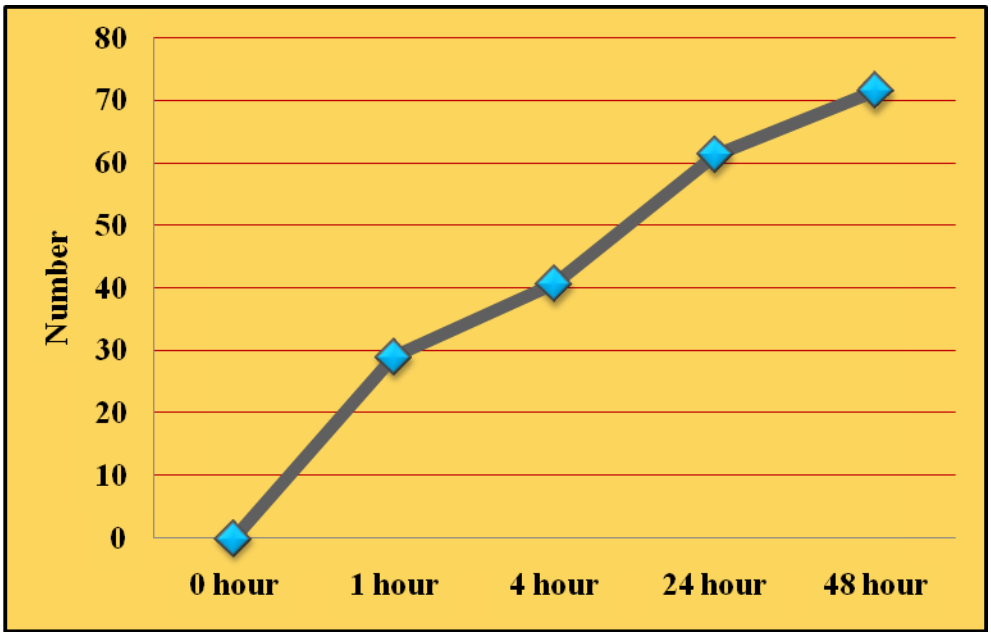
Grpah-2: Mean values of number of viable organisms in group-II at different time interval



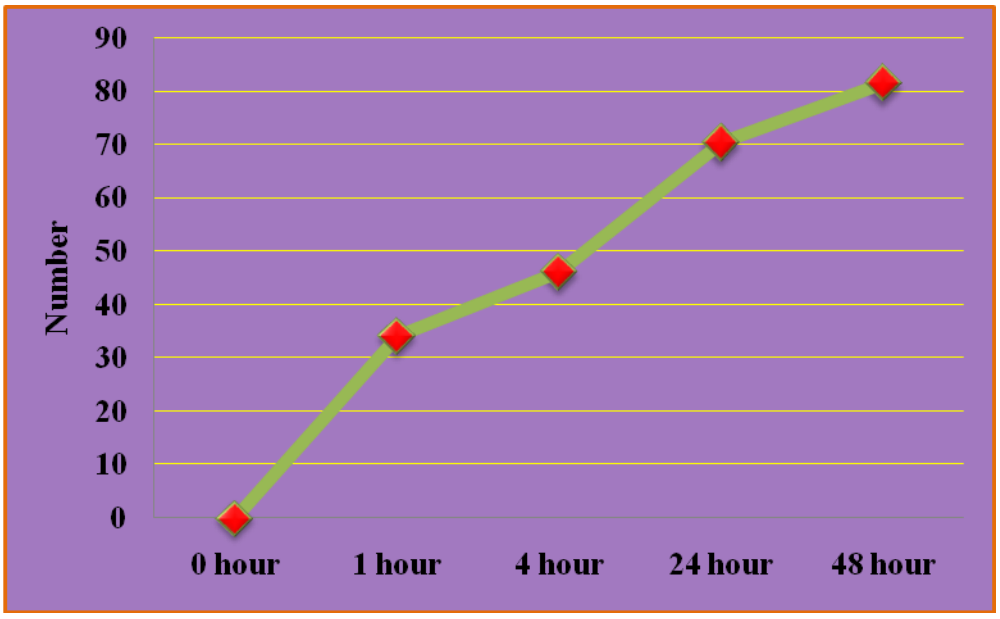
Grpah-3: Mean values of number of viable organisms in group-III at different time interval



Grpah-4: Mean values of number of viable organisms in group-IV at different time interval



Grpah-5: Mean values of number of viable organisms in group-V at different time interval



Grpah-6: Mean values of number of viable organisms in group-VI at different time interval

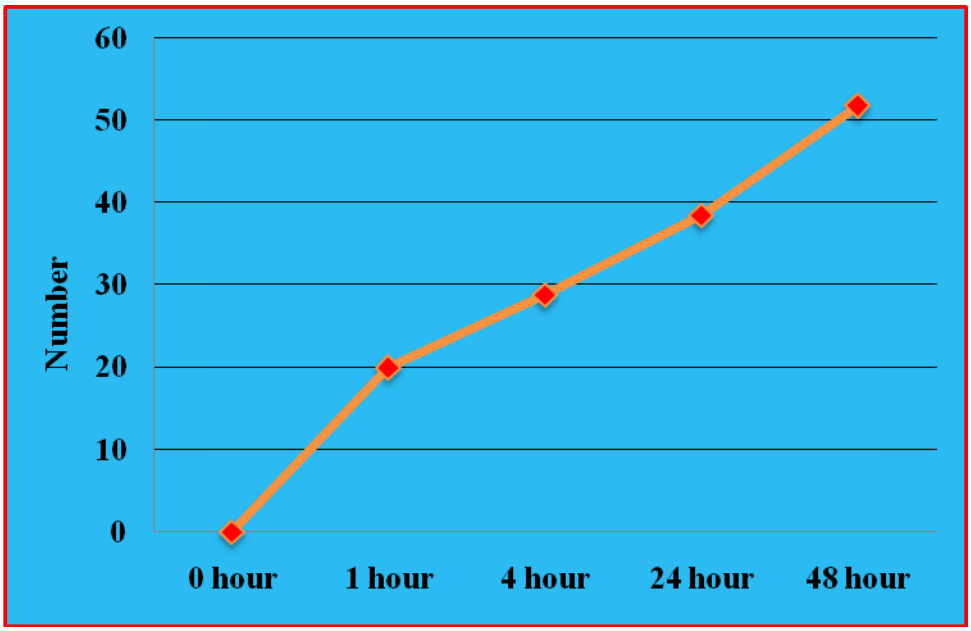


Table-2: Comparison of number of bacterial colonies in group-I with other groups

Groups	O hour (MEAN±SD)	1 hour (MEAN±SD)	4 hour (MEAN±SD)	24 hour (MEAN±SD)	48 hour (MEAN±SD)
Group-I	0.00±0.00	21.33±1.50	62.17±2.32	84.00±1.89	110.17±2.14
Group-II	0.00±0.00	15.00±1.26*	51.50±1.87*	71.83±1.94*	96.17±2.23*
Group-III	0.33±0.82*	31.33±1.97*	57.17±1.83*	78.67±2.42*	91.17±3.06*
Group-IV	0.00±0.00	29.00±2.01*	40.67±1.63*	61.33±1.86*	71.50±2.59*
Group-V	0.00±0.00	34.17±2.71*	46.17±2.23*	70.33±2.33*	81.50±1.87*
Group-VI	0.00±0.00	20.00±1.41	28.84±0.98*	38.50±2.59*	51.83±2.40*

(*P<0.05 significant compared group-I with other groups)

Graph-7: Comparison of number of bacterial colonies in group-I with other groups

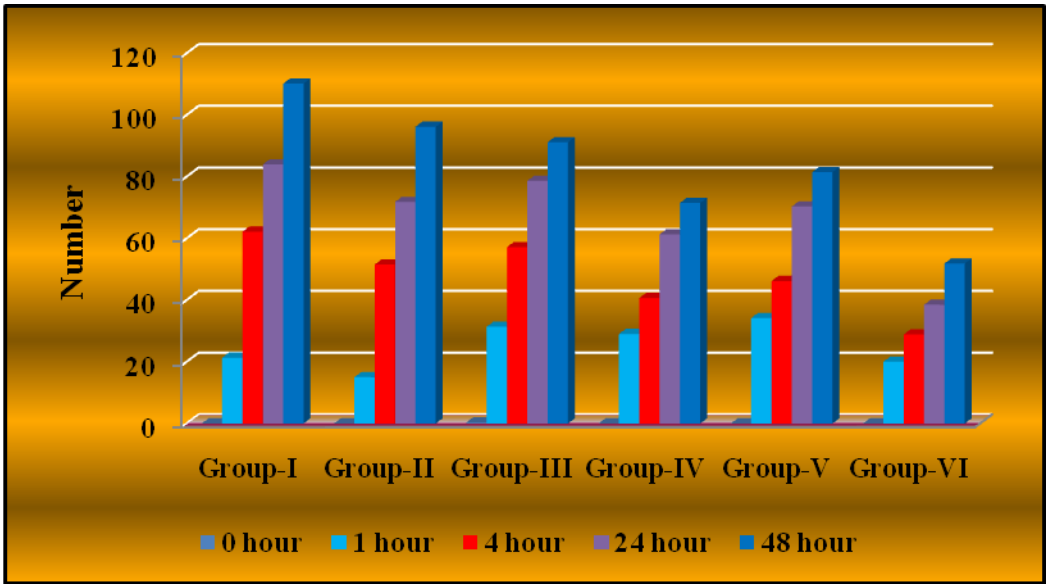


Table-3: Comparison of number of bacterial colonies in group-II with other groups

Groups	O hour (MEAN±SD)	1 hour (MEAN±SD)	4 hour (MEAN±SD)	24 hour (MEAN±SD)	48 hour (MEAN±SD)
Group-II	0.00±0.00	15.00±1.26	51.50±1.87	71.83±1.94	96.17±2.23
Group-I	0.00±0.00	21.33±1.50*	62.17±2.32*	84.00±1.89*	110.17±2.14*
Group-III	0.33±0.82*	31.33±1.97*	57.17±1.83*	78.67±2.42*	91.17±3.06*
Group-IV	0.00±0.00	29.00±2.01*	40.67±1.63*	61.33±1.86*	71.50±2.59*
Group-V	0.00±0.00	34.17±2.71*	46.17±2.23*	70.33±2.33	81.50±1.87*

Group-VI	0.00±0.00	20.00±1.41*	28.84±0.98*	38.50±2.59*	51.83±2.40*

(*P<0.05 significant compared group-II with other groups)

Graph-8: Comparison of number of bacterial colonies in group-II with other groups

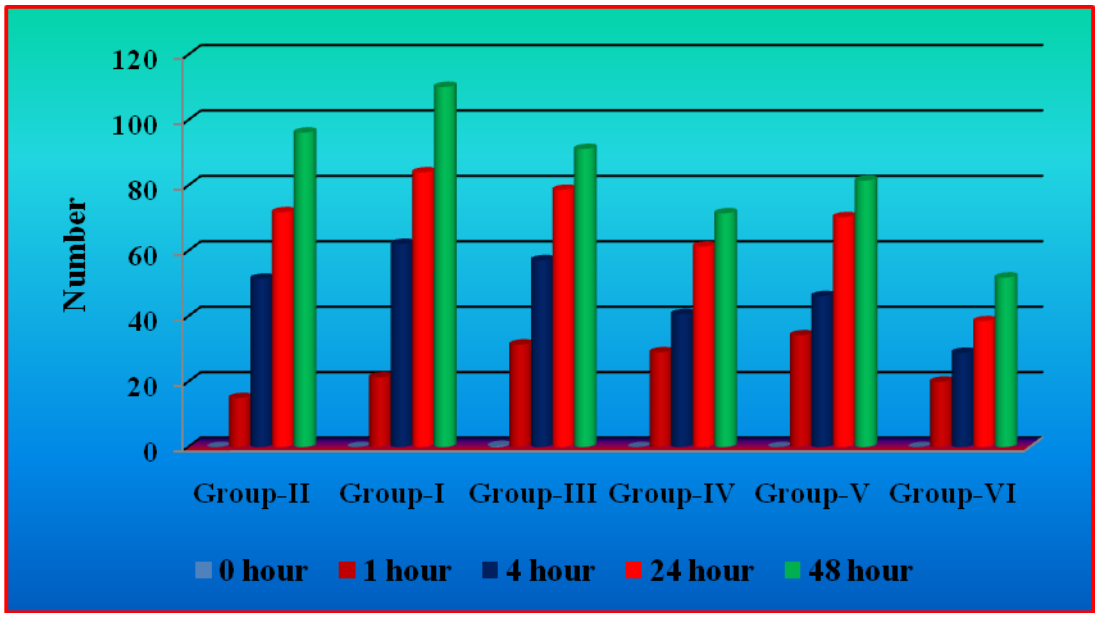


Table-4: Comparison of number of bacterial colonies in group-III with other groups

Groups	O hour (MEAN±SD)	1 hour (MEAN±SD)	4 hour (MEAN±SD)	24 hour (MEAN±SD)	48 hour (MEAN±SD)
Group-III	0.33±0.82	31.33±1.97	57.17±1.83	78.67±2.42*	91.17±3.06
Group-I	0.00±0.00*	21.33±1.50*	62.17±2.32*	84.00±1.89*	110.17±2.14*
Group-II	0.00±0.00*	15.00±1.26*	51.50±1.87*	71.83±1.94*	96.17±2.23*

Group-IV	0.00±0.00*	29.00±2.01*	40.67±1.63*	61.33±1.86*	71.50±2.59*
Group-V	0.00±0.00*	34.17±2.71*	46.17±2.23*	70.33±2.33*	81.50±1.87*
Group-VI	0.00±0.00*	20.00±1.41	28.84±0.98*	38.50±2.59*	51.83±2.40*

(*P<0.05 significant compared group-III with other groups)

Graph-9: Comparison of number of bacterial colonies in group-III with other groups

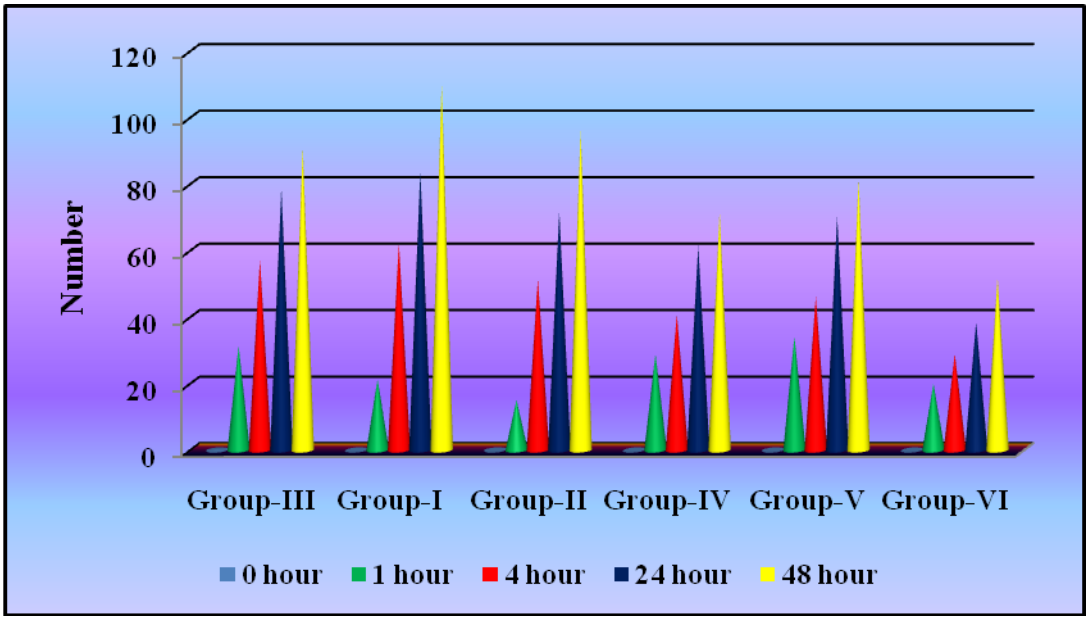


Table-5: Comparison of number of bacterial colonies in group-IV with other groups

Groups	O hour (MEAN±SD)	1 hour (MEAN±SD)	4 hour (MEAN±SD)	24 hour (MEAN±SD)	48 hour (MEAN±SD)
Group-IV	0.00±0.00	29.00±2.01	40.67±1.63	61.33±1.86	71.50±2.59

Group-I	0.00±0.00	21.33±1.50*	62.17±2.32*	84.00±1.89*	110.17±2.14*
Group-II	0.00±0.00	15.00±1.26*	51.50±1.87*	71.83±1.94*	96.17±2.23*
Group-III	0.33±0.82*	31.33±1.97	57.17±1.83*	78.67±2.42*	91.17±3.06*
Group-V	0.00±0.00	34.17±2.71*	46.17±2.23*	70.33±2.33*	81.50±1.87*
Group-VI	0.00±0.00	20.00±1.41*	28.84±0.98*	38.50±2.59*	51.83±2.40*

(*P<0.05 significant compared group-IV with other groups)

Grpah-10: Comparison of number of bacterial colonies in group-IV with other groups

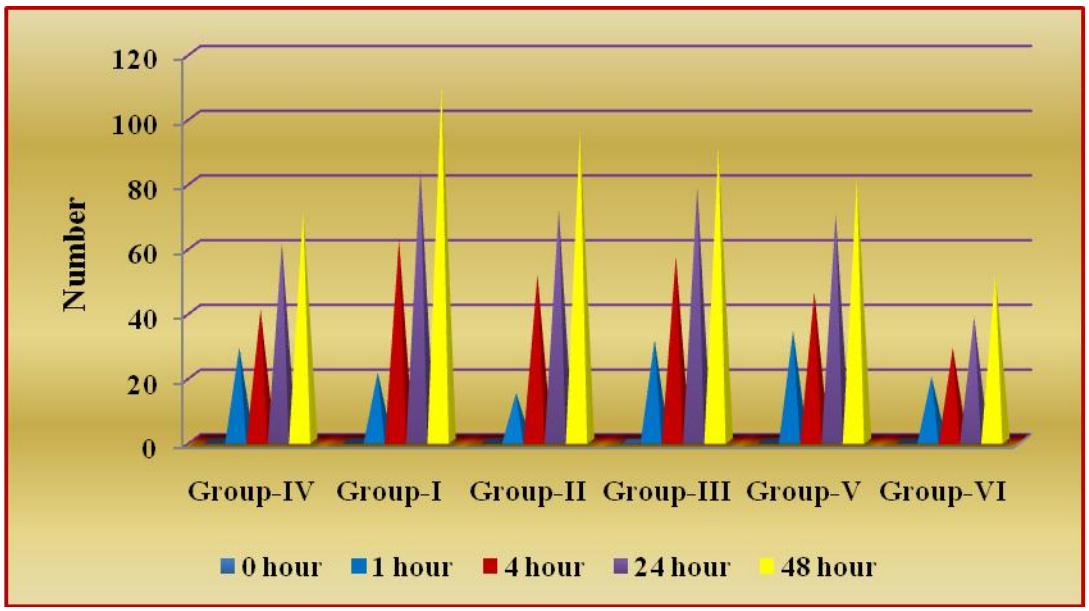


Table-6: Comparison of number of bacterial colonies in group-V with other groups

Groups	O hour	1 hour	4 hour	24 hour	48 hour

	(MEAN±SD)	(MEAN±SD)	(MEAN±SD)	(MEAN±SD)	(MEAN±SD)
Group-V	0.00±0.00	34.17±2.71	46.17±2.23	70.33±2.33	81.50±1.87
Group-I	0.00±0.00	21.33±1.50*	62.17±2.32*	84.00±1.89*	110.17±2.14*
Group-II	0.00±0.00	15.00±1.26*	51.50±1.87*	71.83±1.94	96.17±2.23*
Group-III	0.33±0.82*	31.33±1.97	57.17±1.83*	78.67±2.42*	91.17±3.06*
Group-IV	0.00±0.00	29.00±2.01*	40.67±1.63*	61.33±1.86*	71.50±2.59*
Group-VI	0.00±0.00	20.00±1.41*	28.84±0.98*	38.50±2.59*	51.83±2.40*

(*P<0.05 significant compared group-V with other groups)

Graph-11: Comparison of number of bacterial colonies in group-V with other groups

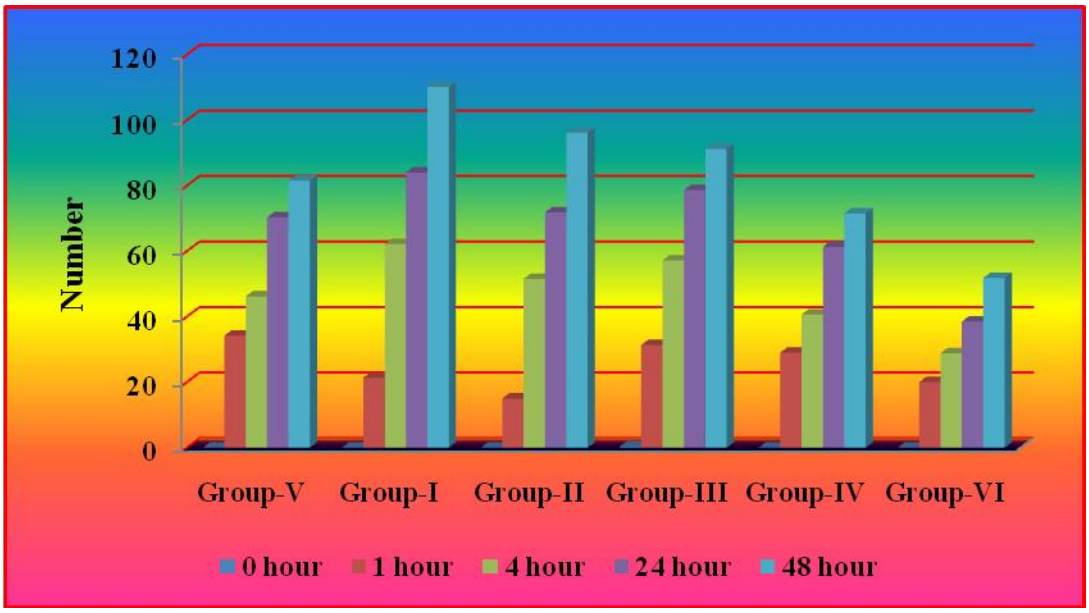


Table-7: Comparison of number of bacterial colonies in group-VI with other groups

Groups	O hour (MEAN±SD)	1 hour (MEAN±SD)	4 hour (MEAN±SD)	24 hour (MEAN±SD)	48 hour (MEAN±SD)
Group-VI	0.00±0.00	20.00±1.41	28.84±0.98	38.50±2.59	51.83±2.40
Group-I	0.00±0.00	21.33±1.50	62.17±2.32*	84.00±1.89*	110.17±2.14*
Group-II	0.00±0.00	15.00±1.26*	51.50±1.87*	71.83±1.94*	96.17±2.23*
Group-III	0.33±0.82*	31.33±1.97*	57.17±1.83*	78.67±2.42*	91.17±3.06*
Group-IV	0.00±0.00	29.00±2.01*	40.67±1.63*	61.33±1.86*	71.50±2.59*
Group-V	0.00±0.00	34.17±2.71*	46.17±2.23*	70.33±2.33*	81.50±1.87*

(*P<0.05 significant compared group-VI with other groups)

Graph-12: Comparison of number of bacterial colonies in group-VI with other groups

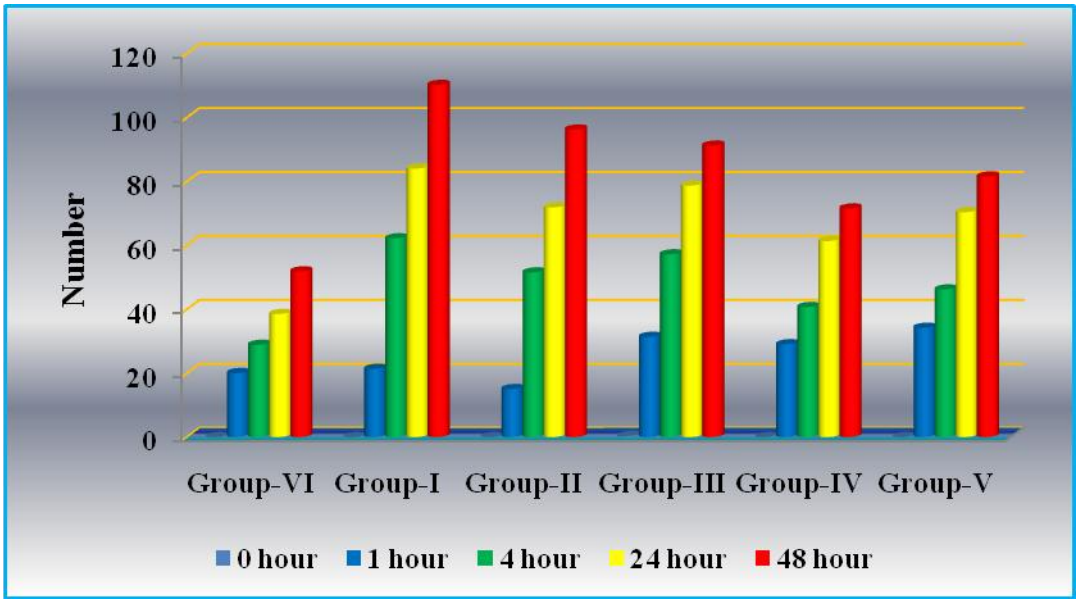


Table-8: Multiple comparisons of number viable organisms of different groups

G	0 hour (MEAN±SD)	1 hour (MEAN±SD)	4 hour (MEAN±SD)	24 hour (MEAN±SD)	48 hour (MEAN±SD)
G-I	0.00±0.00*	21.33±1.50	62.17±2.32	84.00±1.89	110.17±2.14
G-II	0.00±0.00*	15.00±1.26*	51.50±1.87*	71.83±1.94*	96.17±2.23*
G-III	0.33±0.82*	31.33±1.97*,#	57.17±1.83*,#,$	78.67±2.42*,#	91.17±3.06*,#
G-IV	0.00±0.00*	29.00±2.01*,#	40.67±1.63*,#,$	61.33±1.86*,#,$	71.50±2.59*,#,$
G-V	0.00±0.00*	34.17±2.71*,#,†	46.17±2.23*,#,$,†	70.33±2.33*,$,†	81.50±1.87*,#,$,†
G-VI	0.00±0.00*	20.00±1.41#,$,†,‖	28.84±0.98*,#,$,†,‖	38.50±2.59*,#,$,†,‖	51.83±2.40*,#,$,†,‖

(*P<0.05 significant compared Group-I with other groups, #P<0.05 significant compared Group-II with other groups, $P<0.05 significant compared Group-III with other groups, †P<0.05 significant compared Group-IV with other groups, ‖P<0.05 significant compared Group-V with other groups)

Graph-13: Multiple comparisons of number viable organisms of different groups

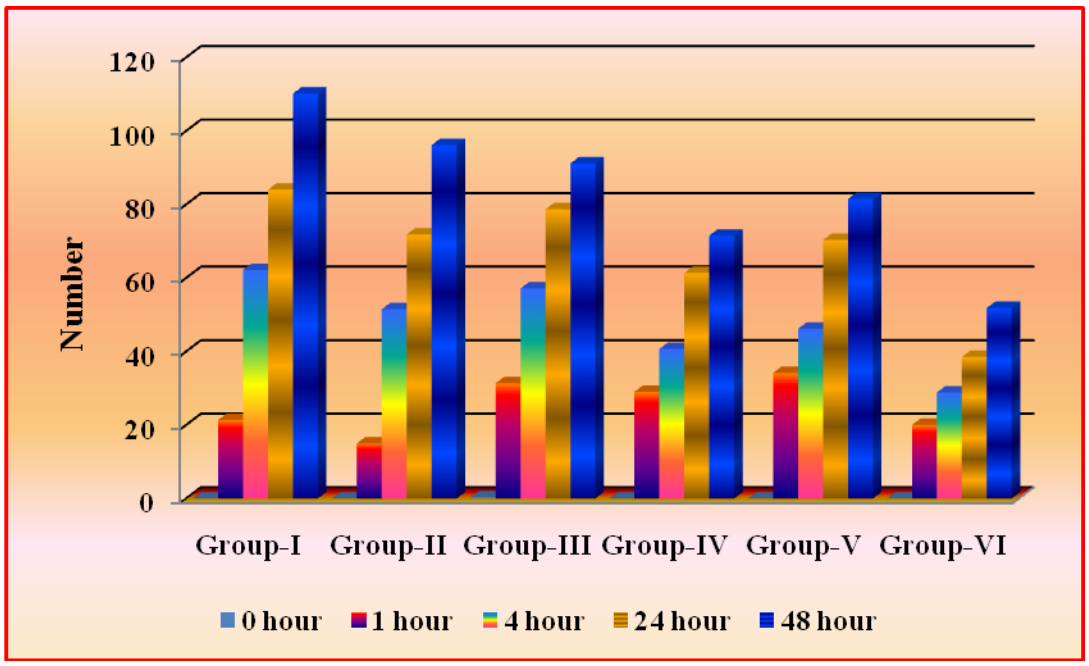


Table-9: Multiple comparison of effect of time on biofilm formation in different groups

Time	Group-I	Group-II	Group-III	Group-IV	Group-V	Group-VI
0 h	0.00±0.00	0.00±0.00	0.33±0.82	0.00±0.00	0.00±0.00	0.00±0.00
1 h	21.33±1.50*	15.00±1.26*	31.33±1.97*	29.00±2.01*	34.17±2.71*,	20.00±1.41*
4 h	62.17±2.32*,#	51.50±1.87*,#	57.17±1.83*,#	40.67±1.63*,#	46.17±2.23*,#	28.84±0.98*,#
24 h	84.00±1.89*,#,$	71.83±1.94*,#,$	78.67±2.42*,#,$	61.33±1.86*,#,$	70.33±2.33*,#,$	38.50±2.59*,#,$
48 h	110.17±2.14*,#,$,‖	96.17±2.23*,#,$,‖	91.17±3.06*,#,$,‖	71.50±2.59*,#,$,‖	81.50±1.87*,#,$,‖	51.83±2.40*,#,$,‖

(*P<0.05 significant compared 0 hour with other time, #P<0.05 significant compared 1 hour with other time, $P<0.05 significant compared 4 hour with other time, ‖P<0.05 significant compared 24 hour with other time)

Graph-14: Multiple comparison of effect of time on biofilm formation in different groups

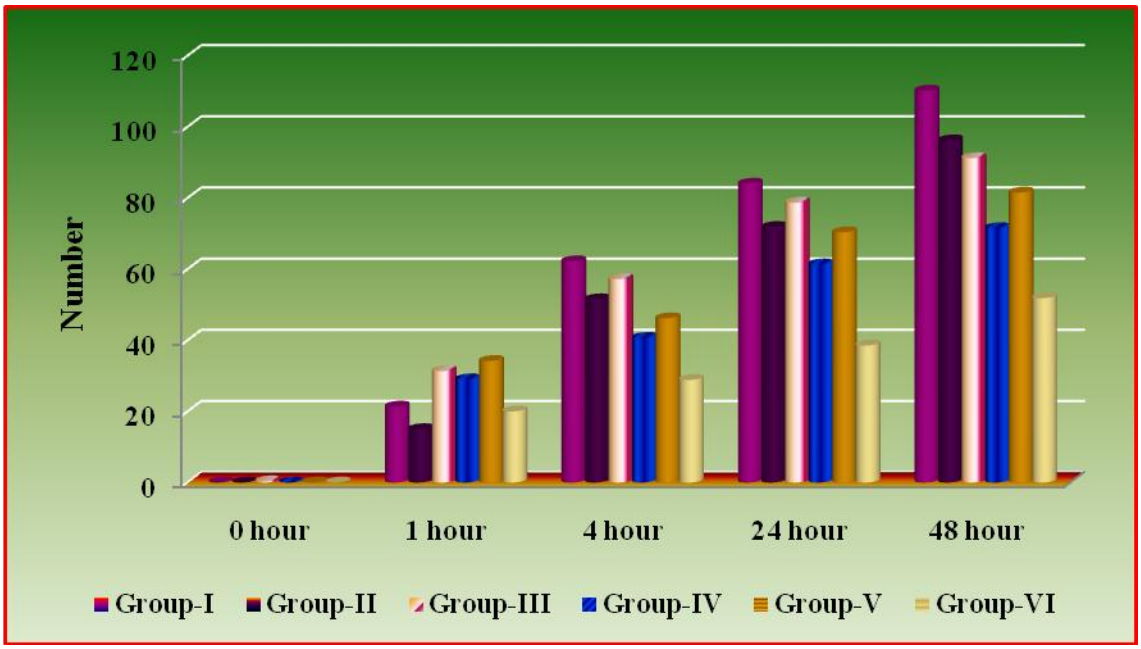


Table (1) shows the mean value of viable organisms of all the six group of samples.

Table (2) compares the number of biofilm formation on group 1 samples with that of the other groups. At zero hour there was no difference in other groups when compared to group3 which was statistically significant ($P<0.05$). This shows that bacterial adhesion on polished samples were significantly less when compared to surfaces modified. In 1 hour there was no significant difference seen in group 6. This shows bacterial adhesion is more on surface modified than that of a polished surface. Where as in 4^{th} hour, 24^{th} hour and 48^{th} hour there was significant difference ($P<0.05$) between other groups. This shows biofilm formation is more on plain samples when compared to surface modified ones.

Table (3) depicts the comparison of number of bacterial adhesion on group 2 samples with other groups. At zero hour there was no significant difference between all other groups when compared to group3 which is statistically significant ($P<0.05$). It was found that there is sequential increase in the number of adhering bacteria. While at zero hour group 2 and group 1 shown almost similar amount of bacteria's. Where as in other at 1^{st} hour, 4^{th} hour, 24^{th} hour and 48^{th} hour there was statistically significant ($P<0.05$) difference when compared to other groups. This shows that gentamicin coating on plain surface is not that effective as coating on a modified surface.

Table (4) shows the comparison of number of adhering bacteria's on group 3 samples with other groups. At Zero hour there was adhering bacteria's when compared to other groups. This shows bacterial adhesion is more on a rough surface. Where as in the 1st hour there is no statistically difference between group 3 and group 6. This explains that there is a native oxide layer of TiO2 which has an anti-bacterial property. In other words the bacterial adhesion at zero hour in surface modified with HA was found to be significantly more probably due to the surface roughness of samples. When compared to group 3 and group 6 samples in 1st hour there was no significant difference. This shows that bacterial adhesion was more on surface modified groups in contrast to group 1 and group2.

Table (5) compares the number of adhering bacteria's on group 4 samples with that of other groups. At zero hour there was no significant difference between all other groups except that of group 3. This shows that due to the action of gentamicin drug bacterial adhesion was prevented when compared to group 3 which is a plain HA treated samples. Where as in 1^{st} hour there was

no significant difference between group 4 and group 3. This again shows that bacterial adhesion was more on surface treated samples. There was significant difference ($P<0.05$) between group 1 and group 2 showing that due to gentamicin bacterial adhesion is prevented. In contrast to group 5 and group 6 there is again statistically significant difference in adhering bacteria's. This shows that anti-bacterial effect is less compared to TiO2.

Table (6) compares the number of bacterial colonies on group 5 samples with that of other groups. Again at zero hour there was no statistically significant difference between all other samples except group 3. This shows anti-bacterial effect of TiO2. At 1st hour there was no statistically significant difference when compared to group 3. This shows bacterial adhesion is delayed due to the anti-bacterial property of TiO2. Rest during all other time intervals, there was significant difference ($P<0.05$). This shows the superior anti-bacterial property of TiO2 when compared to all other groups.

Table (7) depicts the comparison of colonizing bacteria's on group 6 with other groups. At zero hour there was no significant difference between other groups except that of group 3. It's again shows the anti-bacterial property of both gentamicin and TiO2. In the 1^{st} hour there was no difference between group 6 and group 1. This shows bacterial adhesion on surface modified is more when compared to polished surface. In 4^{th} hour, 24^{th} hour and 48^{th} hour there was significant difference ($P<0.05$) when compared to other groups. This gives us a clear idea the addictive effect of both gentamicin and TiO2 as an anti-bacterial agent.

Inference

1. There was no significant difference of bacterial adhesion at zero hour except that of HA blasted samples.

2. In group 1 there is sequential increase in bacterial colonies on plain samples.

3. In group 2 and group 3 Gentamicin coating on plain samples initially retards bacterial adhesion, but due to poor adhesion between gentamicin and polished surface.

4. Initial colonizing of bacteria's are more when compared to polished surface

5. Though initially HA blasted samples retards bacterial adhesion sequential increase of no of bacterial colonies were seen at the rest of the time intervals.

6. Retardation of bacterial adhesion was comparatively better with that of all other samples except with TiO2 blasted samples.

7. Delaying of bacterial adhesion on TiO2 samples was comparatively better than HA blasted surface.

DISCUSSION

In the past 20 years, the number of dental implant procedures has reached steadily about 1 million dental implants per year. The clinical success of oral implant is related to their early osseointegration [32]. Geometry and surface topography with delicate surgical techniques are the prerequisite for a successful clinical outcome[33] . Direct bone apposition on to the surface of titanium is critical for the loading of dental implants. For the successful long term prosthetic rehabilitation, it is necessary to retard bacterial biofilm formation on implant surface, as these bacterial communities are the main source of inflammation of the peri-implant mucosa and bone[34]. Prevention of biofilm formation is always beneficial than the mere attempts to cure the perimplantitis caused by the biofilm because, the treatment of a biofilm is very difficult as the microorganisms are more resistant to antibiotics than their representatives in the planktonic phase. To eliminate the biofilm, a hundred to thousand fold higher antibiotic doses is necessary compared to treatment of planktonic bacteria.

Alumina (Al_2O_3) is frequently used as a blasting material and reduces surface roughness varying with the granulometry of the blasting media [35]. Even though alumina and quartz do not specifically pose any toxicity or biocompatibility issues, these materials are found to be bioinert. Bioactive materials are always the ideal choice in Implantology for better osseointegration. Hydroxyapatite particles play a major role in such situations due to their bioactive nature and the ease in preparation to desired particle sizes which can create surface roughness on the substrate [36]. Grit blasting with HAP is of its first kind and has the advantage of creating surface roughness by impinching HAP moieties on the implant surface thereby modifying it. These impinched particles have better

adhesive strength when subjected to plasma or laser ablation coating of HAP. The bio active coating helps to accelerate the osseointegration which in turn helps the patient for faster rehabilitation. An adherent bio active coating was preferred to have faster bone- implant bonding, thereby reducing the post implantation healing time which provides long term in vivo functionality[37]

Plasma spraying and laser ablation needs complex machines which are expensive. Hence the particular in house method used in the study for the fabrication of sintered HAP along with the freely available and economic sand blasting machine made the technique less complicated[38]. Moreover, the fastness of reproducing roughness on the substrate was found to be better by HAP than laser ablation and plasma coating which made it as one of the material of choice in the present study.

In the present study, tio2 was also selected for modifying the surface of samples as the critical evaluation of various literatures have specified the role of this material in increasing the anchorage of implant. The success rates obtained with dental implants depend upon the volume and quality of the bone. It is often difficult to obtain implant anchorage when the density of bone is less[39].

Comparative clinical studies have shown higher marginal bone levels for TiO2 grit- blasted implants thereby increasing its survival rate[40]. Blasting with TiO2 always show increased adhesion of the particles onto Titanium surface as they are similar metals. This enhances the biomechanical fixation of implants. The antimicrobial property which is an added advantage of TiO2 also was considered in selection of the material for this study[41].

It is always essential to develop implant surfaces that reduce the number of initially adhering bacteria to minimize the plaque formation and subsequent inflammation of the soft tissues. Loading of anti-microbial agent on to the implant

surface is one of its kinds to address the above situation. Anti-microbial drug used in this study was gentamicin since it is effective against gram +ve bacteria[(42)]. Moreover it is one of the few drugs which are heat stable since all the samples are to be autoclaved before doing the biofilm evaluation. Vacuum drying is the technique that is employed in this study to coat the drug on the sample surface[(43)].

A streptococcus sanguis strain was used to evaluate the biofilm formation since streptococcus was the predominant initial colonizing microbes[(44)]. Microbiological study was done to evaluate streptococcal adherence on to the modified and the control samples. An agar plate was used to inoculate the strains.

Six groups of 6 samples each were used for the study. The first group[group 1] had plain polished samples, second group (group 2)had plain samples with gentamicin, third group (group 3) was surface modified with HA, fourth group (group 4)was surface modified with HA and coated with gentamicin, fifth group (group 5) was TiO2 blasted surface and the sixth group (group 6) was gentamicin loaded on TiO2 blasted samples. The study was done at time intervals of 0 hour,1 hour,4 hours,24 hours and 48 hours[(45)].

In group 1, a sequential increase in the number of adhering bacteria onto the substrate was observed. On evaluating, the colony forming units were lesser in this group when compared with group 3,4,5,6 only in the 1st hour. This could be due to the smooth polished surface that inhibits the adhesion of bacteria. The result is in agreement with Cornelius Elter et al who demonstrated through his study that the biofilm formation is more on modified surface than of the polished implants[(46)]. Schreiber et al through his study proved that a titanium surface which is too smooth will prevent cell attachment[(47)]

In the study, it was noted that the amount of CFUs in group 1 were higher in the subsequent test hours. This result can be due to the surface characteristics of titanium. Titanium is covered by a native oxide layer of approximately 2-5nm thick[48] This oxide has amphoteric character and supports cationic and anionic exchange adsorption. When titanium oxides come into contact with the salivary bacteria, primary bonding occurs and adsorption of biopolymer molecules occurs on the surface of the substrate from the sample well. This provides a very reactive surface. This might have lead to the increase in number of bacteria in group 1 samples when compared with other groups[49]

Group 2 presented with a sequential increase in the bacterial adhesion at all the test hours. In comparison with other groups, the microbial colony was considerably less during initial first hour. Later it increased moderately between 4th and 24th hour. The control in the adhesion of bacteria during these hours can be due to the presence of gentamicin. But as the time increases, there can be depletion of the gentamicin layer from smooth surface as the wettability and surface area for adherence of coating is less. Antibiotic coating over polished samples is not effective as coating over a rough surface. This might have led to the increase of bacterial colony during the final hours of test[50].

Group 3 exhibited a sequential increase in the CFUs in all the test hours. When other groups denied growth of bacteria immediately after inoculating with bacteria, HA provided a suitable area for bacterial adhesion even in the 0 hr. the CFUs increased with increase in time in this group of samples. It also showed a considerable increase in the amount of bacterial units when compared with group 4, 5 and 6 in all the test hours. This could be due to the surface irregularities. Various studies have shown that initial colononisation starts from surface

irregularities and spreads out from these areas as a relatively even monolayer of cells. At surface irregularities and other stagnant sites, bacteria, once attached can survive longer. This occurs as they are protected against the naturally occurring removal forces. Roughening of the surface also increases the area available for adhesion[51] Blasting the surface with HA create rough surface which helps in bone attachment but not in bacterial colonization. This could have attributed to the general increase in the bacterial colonization. Though the thin TiO2 layer formed is not sufficient for bacteriostatic activity further adherence occurred after initial retardation of biofilm. Since this type of modification has both surface roughness as well as bioactive layer it is best advisable in areas of pathologic and physical bone loss situation of maxilla and mandible.

Group 4 samples were HA blasted surface with gentamicin coating. The samples showed a gradual increase in the value of bacterial adhesion during the test hours. But the samples presented with a lesser amount of bacterial colonization in comparison with the above groups. The bacterial colonization occurred in this group could be considered moderate at the finishing hours of test. Even though the surface irregularities were more on the HA blasted samples, it paved the way for effective coating of gentamicin on the surface. Anti-biotic coating on rough surface is more when compared to a polished surface since the drug will occupy the pits and craters formed by the roughness during blasting. Since the coated samples were active, local release of gentamicin occurred which minimized the bacterial colonization.

Group 5 samples also showed increase in the amount of bacteria. The adhesion of bacteria was higher than any other groups at the 1st hour. This would be probably due to the surface roughness attained during blasting. This result was in

agreement with the study done by Teughels et al that titanium surface that exhibit rough or hydrophobic(low wettability) surfaces showed high degrees of bacterial colonization[52] In the subsequent test hours, the value was comparable with group 5. This was in agreement with the study conducted by Grossner-Schreiber et al that physical modification of titanium implant surface with TiO2 reduce bacterial adherence [53] than any other surface modifications. The reduction in microbial colonization when compared to groups 1, 2 and 3 could be due to the composition of the implant surface that was blasted with titanium di oxide (TiO2) which increased the thickness of the passivating layer thereby enhancing the inherent antimicrobial activity . Moreover, surface hydrophobicity is a crucial element for influencing the bacterial adhesion. Sanguis is highly hydrophobic organism [54]. The controlled increase in the CFUS would be also due to the thick bacteriostatic surface gained through blasting. This is in agreement with the study of Klaus Gotfredson et al in which he showed that peri-implant health conditions were good in TiO2 blasted surface.

The 6th group which was gentamicin loaded on surface modified with TiO2 showed excellent antimicrobial effect. The values obtained for microbial colonization was much lower when compared with other groups. This might be due to an additive effect of two anti- microbial coating the TiO2 and gentamicin. Bacterial adhesion on this surface was delayed up till 48 hrs. This is in conformation with the studies done by Ivan off C J et al that significant improvement of bone to implant contact occurs in gentamicin coated tio2 blasted surface of titanium in comparison with machined surface [55]. Ivan off et al found on histomorphometric and histological evaluation that all implants were surrounded by a collar of bone to various degrees on surface with TiO2 blasted.

The above results were obtained after the confirmation of surface roughness after blasting with HA and TiO2 using SEM. The SEM results showed satisfactory surface roughness in both the samples. Thus it was be confirmed that these materials could be used for surface treatments in order to enhance bone apposition.

EDAX results assured the probability of using TiO2 for blasting titanium surface as it showed an increased amount of TiO2 on the surface. The study also revealed the significance of using gentamicin coating on implant surface as the biofilm formation was delayed from 4-48 hours on using this drug.

Thus within the scope of the present qualitative study, it could be concluded that The group 6 implants are ideal for patients having poor oral hygiene and a bone quality in type IV bone which is mostly seen in posterior areas of upper jaw . Group 4 implants can be advocated in clinical cases presenting with bone resorption or trauma due to the osteogenetic potential of HA with the antimicrobial effect of gentamicin. Group 1 and group 2 implants should not be used in clinical cases as there is enhanced initial colonization of bacteria which can affect the primary stability thereby leading to failure of implant. Group 3 and group 5 could be used for treatment but will be more effective if used with gentamicin as the initial colonization is by gram positive bacteria.

Tests used to assess the formation of biofilm on the implant surface are not without limitations. To assess the success of implants, evaluation of secondary bacterial colonies should also be done. The interaction of bacterial colonies on the substrate as well as the role of properties of the substrate surface like the wettability and surface free energy which affects adhesion should be studied for a better clinical outcome. The various materials and the methods used for surface

modifications should be considered in future for a better assessment of a successful implant. Above all, to judge the clinical relevance of the present in vitro study, a correlation with a long term in vivo study should be carried out using the same materials.

Intro conclusion.

It has been shown that changes in the physicochemical properties of titanium results in significant modulation of cell recruitment, adhesion, inflammation and bone remodeling activities in addition to regulation on bone formation response. Recently growing micro and nano technology is rapidly advancing the surface engineering in implant dentistry to obtain a successful clinical outcome. This study is a novel approach to unveil the effect of biofilms on implant surface modified by different materials.

Bio film evaluation of surface modified implants with and without gentamicin loaded was evaluated in this study.

The following inferences can be drawn from this study.

1. The SEM analysis and EDAX report of samples showed surface roughness and sufficiently adhered elements Calcium & Phosphorous on HA blasted samples.
2. The SEM results showed surface modifications on TiO2 blasted samples.
3. It is seen that formation of biofilm is seen in all samples.
4. However it is observed that biofilm was delayed in surface modified and gentamicin loaded samples.
5. Gentamicin loaded on TiO2 surface showed low concentrations of biofilm formation among all the other 5 groups.
6. It is noticed within 1 hour Bio film formation was on plain polished surface.
7. However bio film formation was delayed more than 1 hour on plain polished gentamicin loaded samples.
8. In contrast the biofilm formation was delayed on TiO2 blasted surface even up to 48 hrs.
9. In contrast in HA treated implants it was delayed only up to 4 hrs.

From the above findings it can be concluded that implant surface modified with TiO2 and gentamicin showed delayed biofilm formation even up to 48 hrs. These implants can retard the plaque formation thus prevents peri-implantitis in the primary healing stage. This in turn can prevent failure of implants. This is ideal in situations where the patient is

having poor bone quality, poor oral hygiene and in patients suffering from debilitating disease.

Surface modification with HA has gained considerable osteoconductive surface which is a boon for the production of future implants with less expense, however further studies are to be carried out to prove its efficacy.

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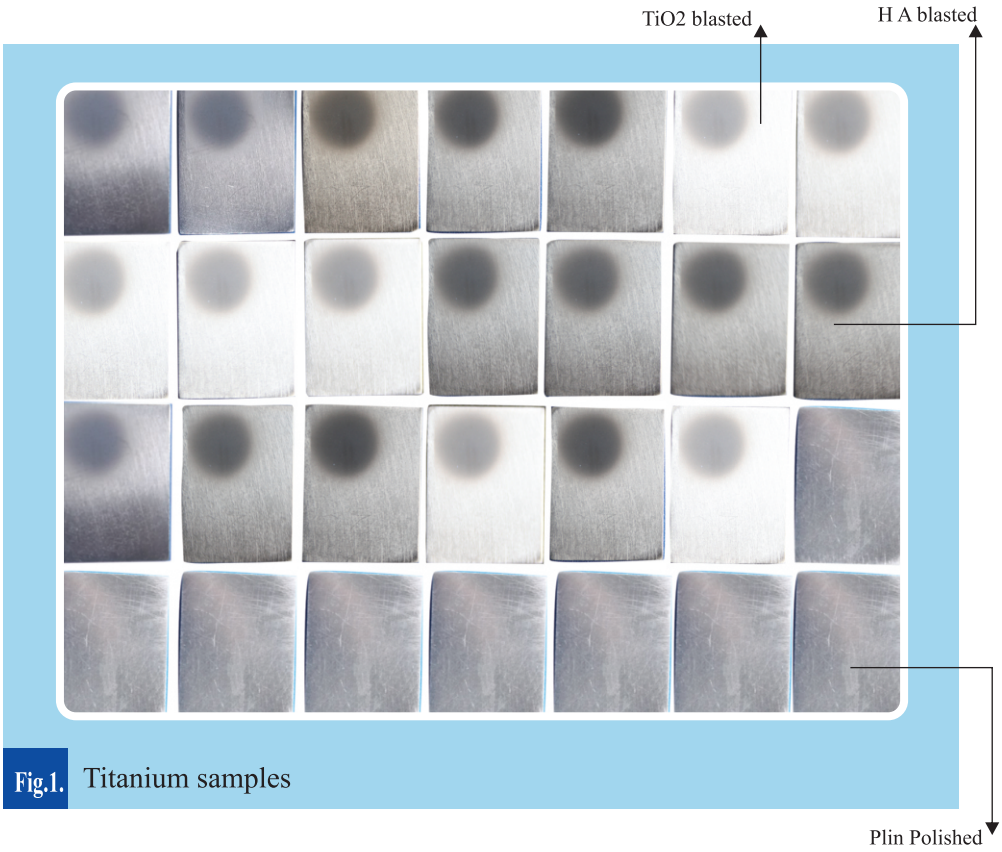

Fig.1. Titanium samples

Fig.2. Sintered hydroxyapatite

Fig.3. Sintered TiO2

Fig.4. Gentamycin

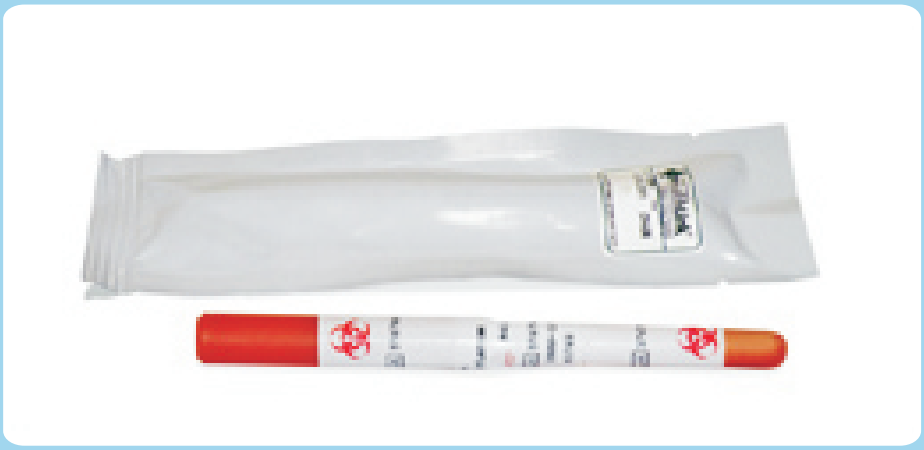

Fig.5. Microbial Strain

Fig.6 Titanium dioxide

Fig.7. Phosphate Buffer Saline

Fig.8. Ringer Solution

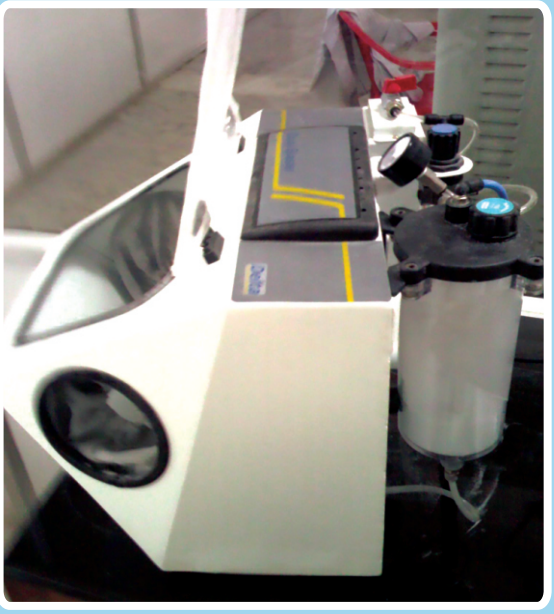

Fig.9. Sandblaster

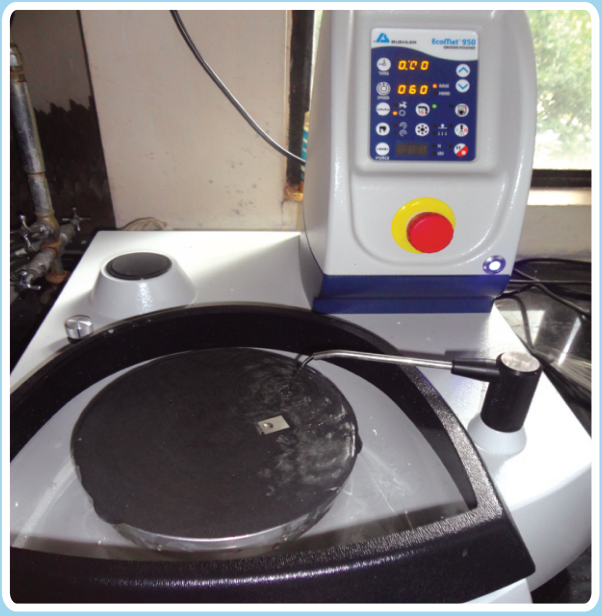

Fig.10. Grinder and polisher

Fig.11. Isostatic pressing machine

Fig.12. Pulverizer

Fig.13. Tumbling machine

Fig.14. Ultrasonic cleaner

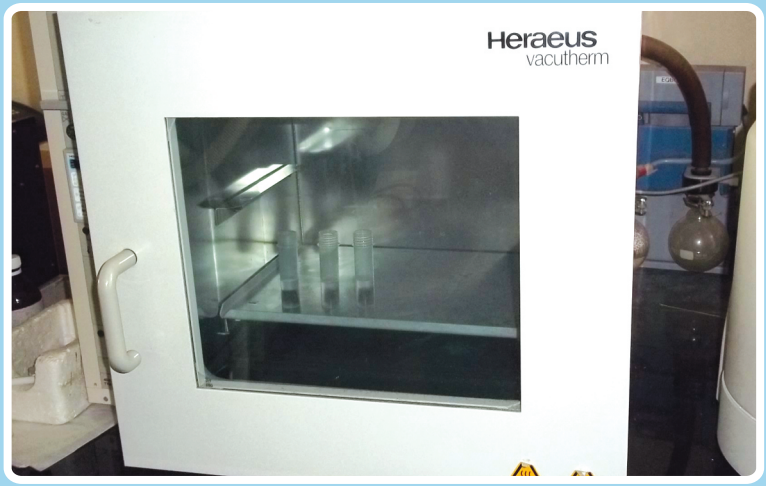

Fig.15. Vacuum dryer

Fig.16. Auto clave

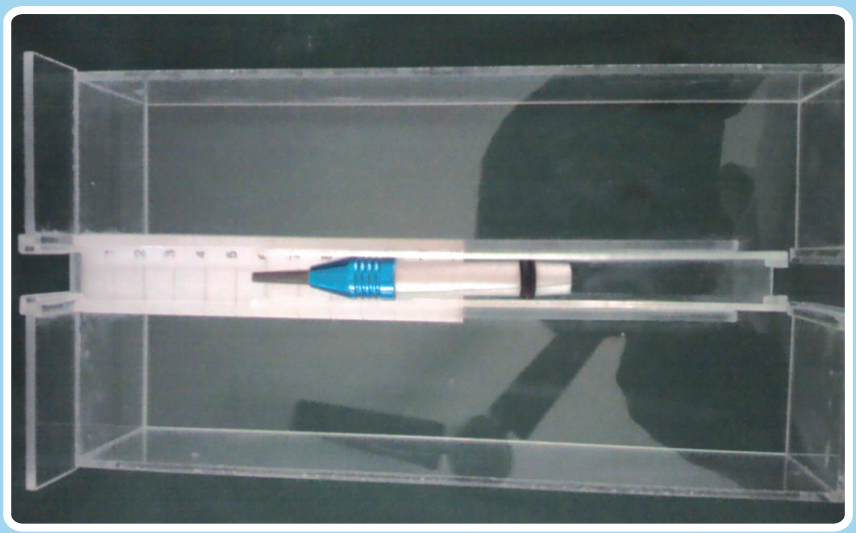

Fig.17. Gauge and blasting gun holder

Fig.18. Sieves

Fig.19. Micro polish

Fig.20. Centrifugal Tube

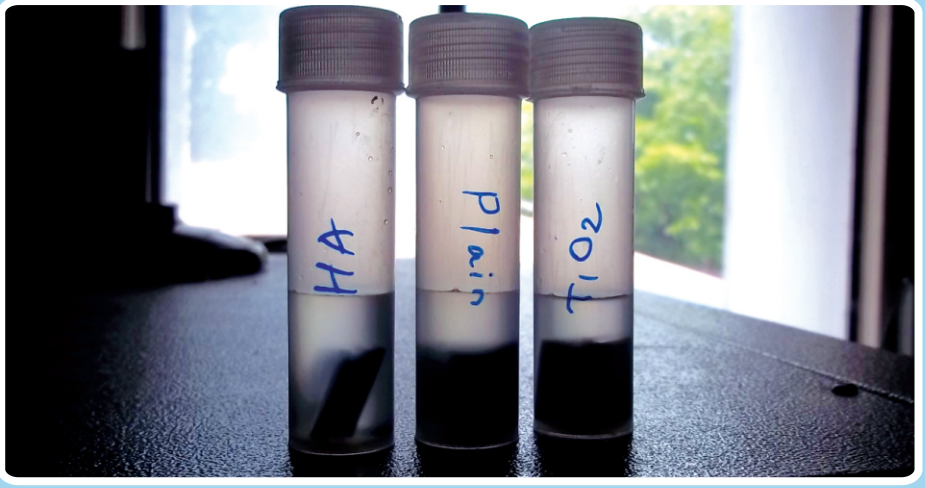

Fig.21. Test tube

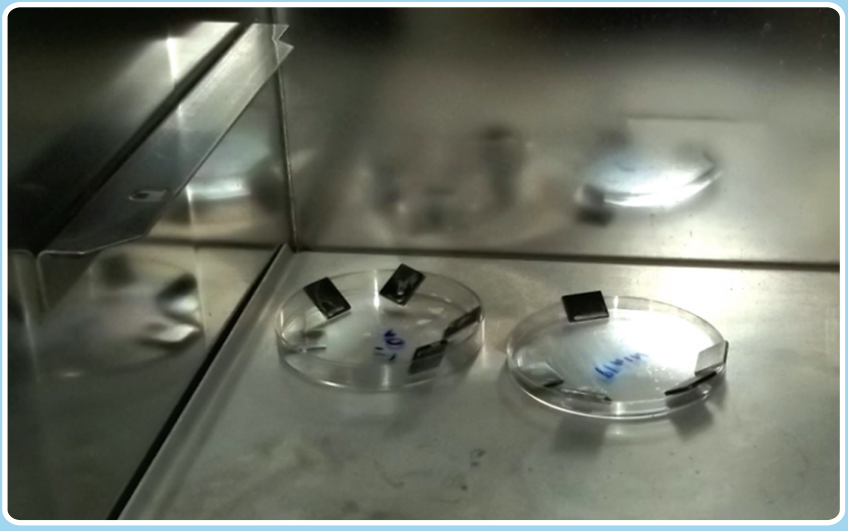

Fig.22. Petri dish

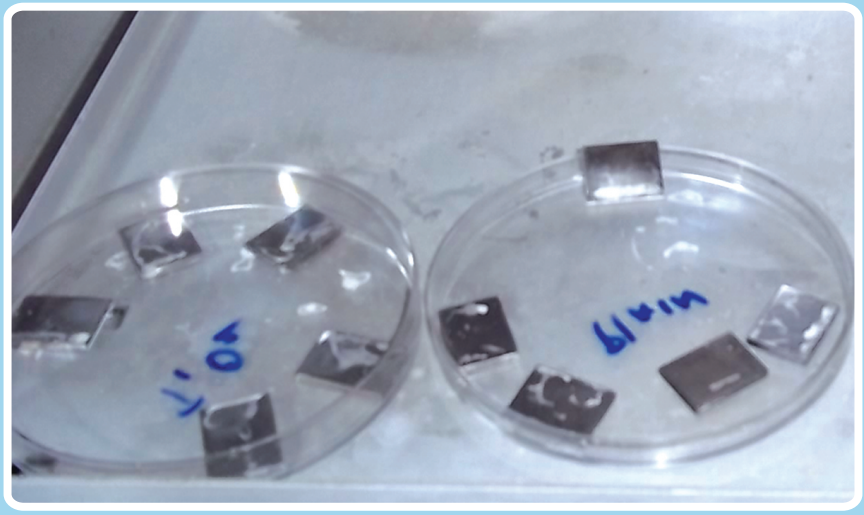

Fig.23. Gentamycin Loaded Samples

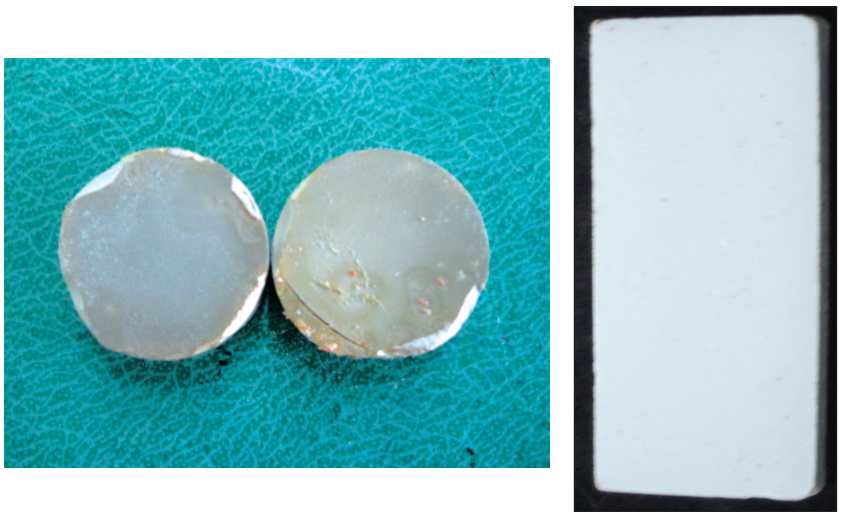

Fig.24. Sintered HA & TiO2 blocks

Fig.25. SEM & EDAX

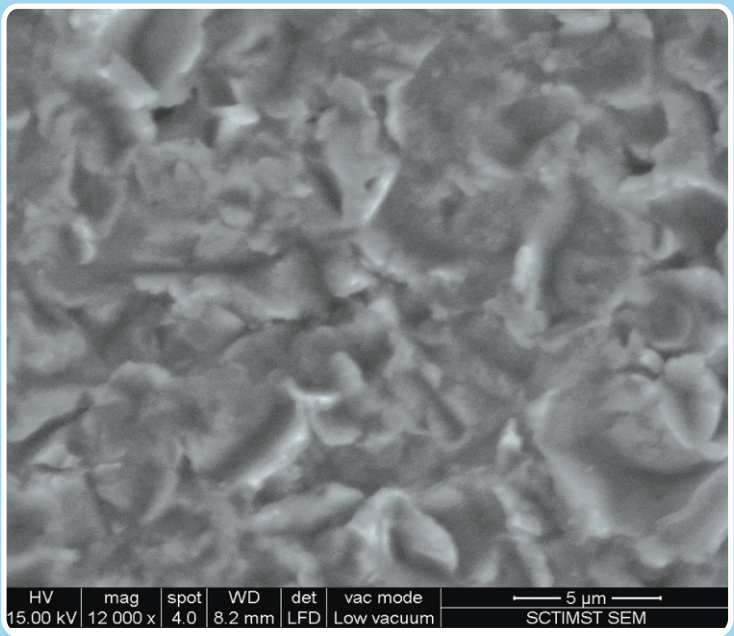

Fig.26. SEM Images of TiO2 Blasted Samples

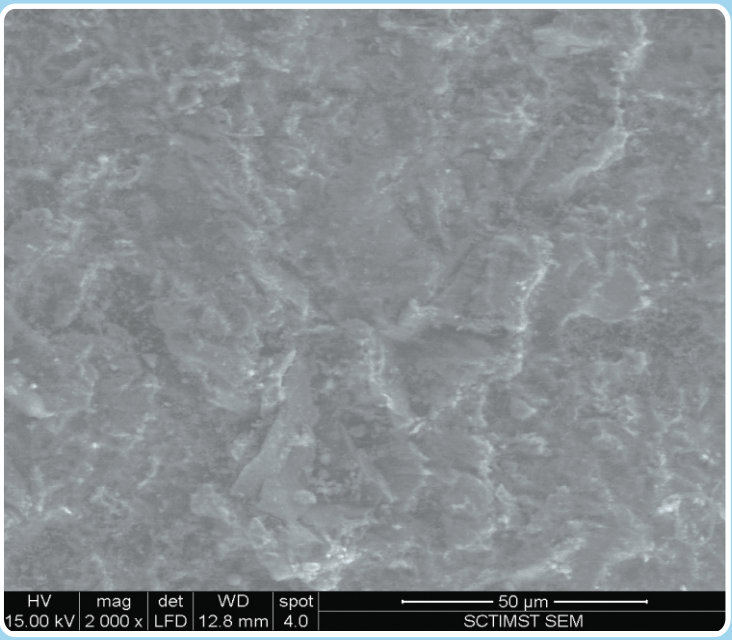

Fig.27. SEM image of HA blasted sample

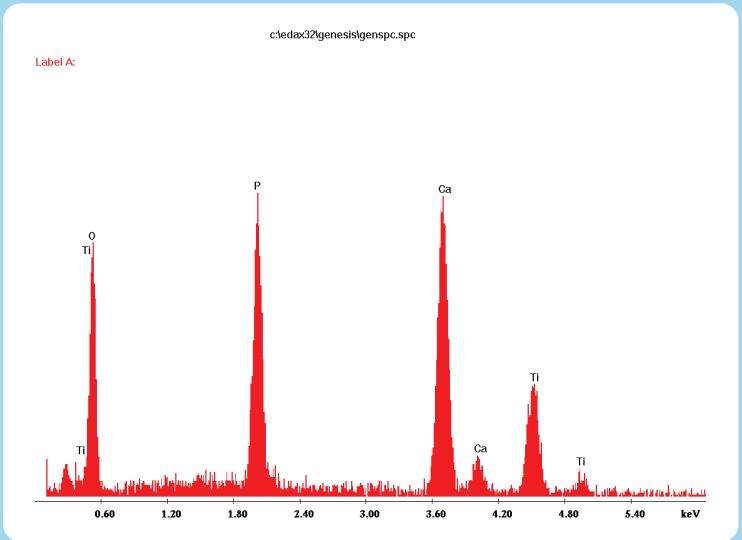

Fig.28. EDAX of HA blasted sample

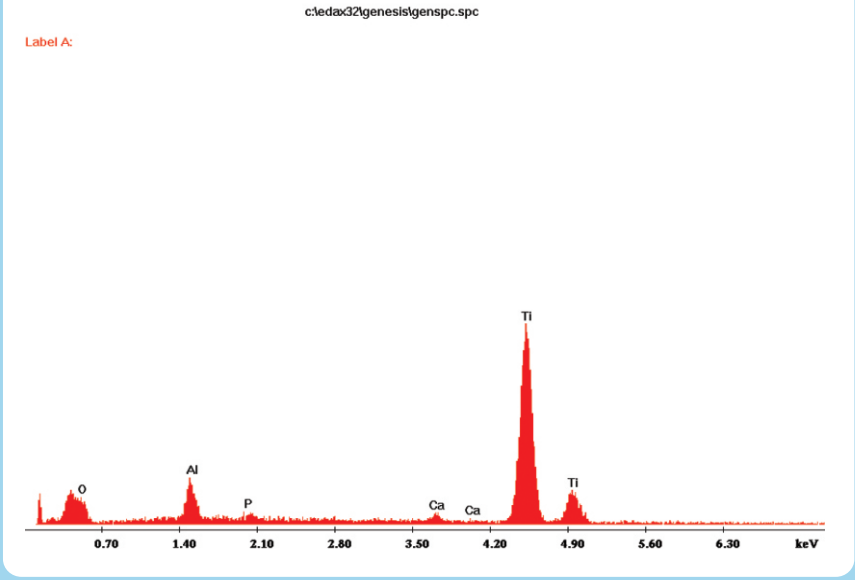

Fig.29. EDAX of TiO2 blasted sample

EDAX ZAF Quantification (Standardless)
Element Normalized
SEC Table : Default

Elem	Wt %	At %	K-Ratio	Z	A	F
O K	63.52	80.40	0.1125	1.0271	0.1724	1.0001
P K	12.68	8.29	0.0845	0.9592	0.6902	1.0065
CaK	15.20	7.68	0.1396	0.9620	0.9414	1.0143
TiK	8.60	3.63	0.0677	0.8836	0.8914	1.0000
Total	100.00	100.00				

Element	Net Inte.	Backgrd Inte.	Error	P/B
O K	8.34	0.21	3.55	39.71
P K	12.25	0.53	2.98	23.11
CaK	16.68	0.24	2.48	69.50
TiK	6.94	0.13	3.87	53.38

c:\edax32\genesis\genspc.spc
Label :
Acquisition Time : 16:11:46 Date : 2-Sep-2011

kV: 30.00 Tilt: 0.00 Take-off: 35.00 AmpT: 51.2
Det Type:SUTW, Sapphire Res: 130.27 Lsec: 100

Fig.30. EDAX Report of HA Blasted Samples